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The Action of Isoniazid on the Transaminases of *Mycobacterium tuberculosis* (B.C.G.)

By JEAN YOUATT
Bacteriology Department, University of Melbourne, Victoria, Australia

(*Received* 3 *May* 1957)

One of the proposed mechanisms for the action of *iso*nicotinic acid hydrazide (isoniazid) has been the inhibition of enzyme systems which require pyridoxal phosphate (Cymerman-Craig, Rubbo, Willis & Edgar, 1955). However, it appeared unlikely that the inhibition of an enzyme system present in many bacteria could account for the highly specific activity of isoniazid in low concentrations against species of *Mycobacterium*.

Sakai (1954) found that the aspartic–glutamic acid transaminase of *Mycobacterium tuberculosis* B.C.G. strain (B.C.G.) was inhibited 90–100% by 0·01 M-isoniazid with an isoniazid-sensitive strain, whereas the transaminase of the resistant B.C.G. was not inhibited. Transamination of asparagine, glycine, phenylalanine, leucine, valine, alanine and aspartic acid by avian tubercle bacilli was described by Ito & Sugano (1954), who also observed the inhibition of the reaction by 0·01 M-isoniazid. Yonemura & Iisuka (1955) described the transamination of twenty amino acids in the presence of α-oxoglutarate, by a virulent strain of bovine tubercle bacilli. They found no transamination between any amino acid, other than glutamic acid, and pyruvic or oxaloacetic acid.

Yamamoto (1955) found that cysteine desulphydrase of sensitive and resistant strains of B.C.G. was inhibited by isoniazid. The inhibition was partially reversed by pyridoxal and adenosine triphosphate.

The results with enzymes of *Mycobacterium* closely parallel those obtained with organisms naturally insensitive to isoniazid, such as *Escherichia coli*, with which it was shown that enzymes requiring pyridoxal phosphate were inhibited by isoniazid at concentrations of the order of 0·01 M (Yoneda, Kato & Okajima, 1952).

The experiments described below were made with the aim of determining whether the inhibition of transaminase in *Mycobacterium* could account for the highly specific action of isoniazid in low concentrations.

METHODS

Cultures. Surface cultures of B.C.G. were grown on Sauton medium (Soltys, 1952) to which 3 g. of casein hydrolysate (British Drug Houses Ltd.) was added/l. Dispersed cultures were grown in Sauton medium containing 0·05% of polyoxyethylene sorbitan mono-oleate (Tween 80). Suspensions of the organisms in pentane were used to inoculate the surface of the medium. Subcultures were made at intervals of one week and cultures were incubated at 37° for 7–14 days.

A culture of B.C.G. resistant to isoniazid was obtained by surface inoculation of Sauton medium containing 1 μg. of isoniazid/ml. In this first subculture growth was slow but thereafter the organisms grew readily in successive transfers to media with 10, 20 and 40 μg. of isoniazid/ml.

Preparation of suspensions and extracts. Surface cultures were suspended in 0·9% NaCl by gentle grinding in a Potter–Elvehjem homogenizer. This treatment resulted in some cell damage, as evidenced by leakage of cell contents into the suspending medium. Cell-free extracts were prepared by shaking suspension for 2 min. in a Nossal disintegrator (Nossal, 1953). Dialysis of the extracts overnight against distilled water at 5° was essential to remove the amino acids, of which glutamic acid, aspartic acid and alanine were consistently detected by paper chromatography. Tyrosine was also found in most cell extracts. These internal free amino acids were also found when the cultures were grown with ammonium chloride as their only source of nitrogen.

Detection and estimation of transaminase activity. Unless otherwise stated, transaminase activity was measured under the following conditions. The final concentrations of the reagents, which were adjusted to pH 8, were: keto acid, 0·05 M; amino acid, 0·025 M; Sørensen's phosphate buffer, 0·1 M; cell suspensions, 20–50 mg. (dry wt.)/ml. and cell extracts, 10–20 mg. (dry wt.)/ml. A volume (1 ml.) of the reaction mixture was incubated at 35° for the periods indicated in the tables, after which 1·5 ml. of 0·67 M-acetate buffer, pH 4, was added. After centrifuging, 2 ml. of supernatant was transferred to a Warburg flask with 0·5 ml. of a suspension of *Proteus mirabilis* in the side arm. Carbon dioxide production was measured at 35°. The use of *Proteus* suspensions for this purpose is described below.

In qualitative examinations the supernatants were spotted on Whatman no. 1 paper and chromatograms were run in butanol–acetic acid–water (4:1:1) and developed with ninhydrin in butanol (0·25%, w/v).

Estimation of glutamic acid with Proteus mirabilis. *P. mirabilis* was grown on meat-infusion agar enriched with freshly prepared yeast extract and 0·1% sodium glutamate. The organism was not dangerous to handle, could be grown on a simple medium with yields up to 4 g. dry wt./l. and had a glutamic acid decarboxylase activity of the order of 0·4 μmole of glutamic acid/hr./mg. dry wt., at 37° and pH 4·5. The following methods of preparation gave suspensions of satisfactory specificity: 1, lyophilized suspensions resuspended in buffer at pH 4; 2, suspensions at pH 4 which had been shaken with glass beads in the Nossal

disintegrator for 1 min.; 3, freshly harvested suspensions with 0·03 M-isoniazid. (Freshly harvested suspensions showed production of CO_2 in the absence of added glutamic acid. This was suppressed by the presence of isoniazid.) Cell-free extracts could be prepared from 2 by centrifuging. None of these preparations showed gas production with L-glutamine, DL-aspartic acid, L-ornithine, DL-lysine, DL-arginine, DL-tyrosine, DL-histidine, or α-oxoglutaric, pyruvic or oxaloacetic acid, or with these keto acids in combination with DL-aspartic acid, DL-valine, DL-leucine, DL-isoleucine, DL-methionine, DL-alanine, DL-phenylalanine. One molecule of CO_2 was produced for each molecule of glutamic acid and γ-aminobutyric acid was identified by paper chromatography. The decarboxylase was not inhibited by isoniazid at 0·1 M in either cell suspensions or extracts.

Requirement for pyridoxal phosphate. The addition of pyridoxal phosphate to suspensions and extracts of B.C.G. was found to be unnecessary and it was omitted from all experiments except the surveys of the range of transaminase activities and experiments where the reversal of isoniazid inhibition was measured. The pyridoxal phosphate of the aspartic–glutamic acid transaminase of B.C.G. appears to be firmly bound to the apoenzyme. Stimulation by pyridoxal phosphate was observed only when the enzyme had been precipitated with saturated ammonium sulphate and dialysed against acetate buffer pH 4 for several days. The protein was precipitated at this pH but redissolved in buffer at pH 8. The addition of pyridoxal phosphate to this preparation caused a 50% increase in activity. Halpern & Grossowicz (1956) have recently described the stability of the transaminase of *Mycobacterium phlei*.

Use of isoniazid as an inhibitor. Unless otherwise stated, suspensions and extracts were pre-incubated with isoniazid for 30 min. before the addition of the substrates. Prolonged exposure of the cells to isoniazid before harvesting was also tested.

RESULTS

Range of transaminase activities

Each of the following amino acids was incubated with α-oxoglutaric acid, pyruvic acid and oxaloacetic acid in the presence of cell suspensions and extracts with pyridoxal phosphate: DL-alanine, L-asparagine, DL-aspartic acid, L-arginine, L-cysteine, DL-cystine, L-glutamic acid, L-glutamine, glycine, DL-histidine, DL-hydroxyproline, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, DL-proline, DL-serine, DL-threonine, DL-tyrosine, DL-tryptophan, DL-valine. By the use of paper chromatography, evidence of transaminase activity was found in the following systems: α-oxoglutarate and the amino acids, aspartic acid, leucine, isoleucine, valine, asparagine, alanine, methionine, and phenylalanine; pyruvic acid and glutamic acid; oxaloacetic acid and glutamic acid. Asparagine is rapidly metabolized by the organisms to aspartic acid and may not be directly concerned in the transaminase reaction. No variation in the range of transaminase activity was found with the age of the culture or with its resistance to isoniazid.

Inhibition of transaminases by isoniazid

Differences in the degree of sensitivity of transaminases to isoniazid are shown in Table 1. Even with incubation for 3 hr. (*B*) transamination of alanine was barely detectable. The reaction was shown to be linear with time, over 1½ hr. (*A*). Where there is overlap between the two series it will be seen that the only change in the pattern of behaviour was a small increase in the sensitivity to isoniazid of the aspartic–glutamic acid transaminase. No differences were detected between sensitive and resistant B.C.G. greater than were found with different preparations of sensitive organisms.

Isoniazid increased the leakage of glutamic acid from whole-cell suspensions. It was therefore not possible to determine the inhibition of the transaminase reaction with whole cells.

Table 1. *Sensitivity to isoniazid of different transaminase reactions in extracts of isoniazid-sensitive B.C.G.*

Reaction mixtures: α-oxoglutarate 0·05 M, amino acid 0·025 M, phosphate buffer 0·1 M, all at pH 8. Extracts of sensitive B.C.G. 20 (*A*) and 10 (*B*) mg. dry wt./ml. Pre-incubated with isoniazid for 30 min. at 35°. Reaction time: (*A*) 1½ hr.; (*B*) 3 hr.

	Amino acid	Rate of transamination (μmoles of glutamic acid/hr./ml.)	% inhibition by isoniazid		
			0·1 M	0·01 M	0·001 M
(*A*)	Aspartic acid	3·3	74	35	8
	Leucine	5·8	100	100	52
	Isoleucine	4·82	100	100	54
	Valine	4·42	100	100	55
(*B*)	Aspartic acid	1·74	94	49	27
	Leucine	2·1	100	100	40
	Isoleucine	3·74	100	89	43
	Valine	3·45	98	94	53
	Alanine	0·13	100	47	8
	Phenylalanine	0·77	69	22	0
	Methionine	0·8	89	66	45

Sakai (1954) reported that the aspartic–glutamic acid transaminase of B.C.G. was 90–100% inhibited by 0·01 M-isoniazid, whereas in these experiments less inhibition was observed. An explanation of the discrepancy was found in the concentration of keto acid employed. Sakai used α-oxoglutarate at a concentration of 0·005 M. Table 2 shows that the rate of transamination is very sensitive to changes in substrate concentration and the degree of inhibition depends on the relative concentrations of isoniazid and keto acid.

Above 50 μmoles of α-oxoglutarate/ml. the enzyme is approaching saturation and the degree of inhibition by a given concentration of isoniazid is independent of the concentration of keto acid. Table 3 shows the inhibition by a range of isoniazid concentrations with two concentrations of α-oxoglutarate. From the linear plot of log [isoniazid] against the percentage inhibition the concentration of isoniazid producing 50% inhibition was in each case 30 μmoles/ml.

Effect of hydrazones of isoniazid on transaminase

Davison (1956) described the formation of a hydrazone by isoniazid and pyridoxal phosphate and was able in this way to explain the inhibition of enzymes requiring pyridoxal phosphate. Thus it would be expected that the addition of pyridoxal phosphate would bring a reversal of isoniazid inhibition and that stable hydrazones of isoniazid should not inhibit the transaminase reaction. In the present study it was found that an equimolar concentration of pyridoxal phosphate completely antagonized the isoniazid inhibition of transaminase, and this antagonism was also observed to a lesser degree (30%) with pyridoxine and adenosine triphosphate. Acetylacetone *iso*nicotinoylhydrazone and the isoniazid hydrazone of pyridoxal produced no inhibition of the isoleucine–glutamic transaminase at 0·01 M, at which concentration isoniazid inhibited 90–100%. The isoleucine–glutamic transaminase was selected for this experiment because of its greater sensitivity to isoniazid than the aspartic–glutamic transaminase.

The failure of the hydrazones to inhibit the transaminase reaction was of particular interest because both of these compounds had a tuberculostatic activity similar to isoniazid (Table 4). The hydrazones were stable in the presence of heavy suspensions of B.C.G. in Sauton medium. With picryl chloride as a reagent for isoniazid (Cuthbertson & Ireland, 1952) free isoniazid could be detected with certainty at 0·001 M. The hydrazones were present at 0·025 M. The test was therefore capable of detecting the release of free isoniazid down to 4% of the theoretical isoniazid. The tuberculostatic activity of the hydrazones could not be explained on the grounds of the release of

Table 2. *Effect of keto acid concentration on the rate of transamination and the degree of inhibition by isoniazid*

Reaction mixtures: α-oxoglutarate concentrations as indicated in the table, DL-aspartic acid 0·025 M, phosphate buffer 0·1 M, all at pH 8. In series (*A*) isoniazid was present at 8·3 μmoles/ml. and in series (*B*) at 25 μmoles/ml. An extract of sensitive B.C.G. was used (10 mg. dry wt./ml.). Pre-incubated with isoniazid for 30 min. at 35°. Reaction time 1½ hr. at 35°.

	α-Oxoglutarate (μmoles/ml.)	Glutamic acid produced (μmoles/ml./hr.) Without isoniazid	With isoniazid	% inhibition
(*A*)	100	10·2	6·7	34
	50	6·7	4·7	30
	25	4·8	3·6	25
	12·5	2·5	1·8	28
	6·25	0·55	0·0	100
(*B*)	100	6·0	4·4	27
	50	5·8	3·7	38
	25	4·8	2·1	56
	12·5	2·9	0·17	94
	6·25	0·67	0·0	100

Table 3. *Inhibition of transaminase by varying concentrations of isoniazid, with constant α-oxoglutarate concentration*

Reaction mixture: α-oxoglutarate, 50 and 70 μmoles/ml. respectively in (*a*) and (*b*); DL-aspartic acid, 0·025 M; phosphate buffer, 0·1 M; extract of sensitive B.C.G. 10 mg. dry wt./ml. All reagents were at pH 8. Pre-incubated with isoniazid for 30 min. at 35°. Reaction time, 3 hr. at 35°.

Isoniazid (μmoles/ml.)	% inhibition (*a*)	(*b*)
90	—	75
80	—	73
70	71	66
60	70	—
50	65	66
30	60	—
20	—	37
10	22	22
5	10	9

Table 4. *Tuberculostatic concentrations of isoniazid and its hydrazones*

Compound	Tuberculostatic end-point*
Isoniazid	5×10^{-7} M
Acetylacetone *iso*nicotinoylhydrazone	2×10^{-6} M
Pyridoxal *iso*nicotinoylhydrazone	10^{-6} M

* The lowest concentration of isoniazid which prevented growth of *Mycobacterium tuberculosis* H 37 Rv in Youman's medium after 14 days' incubation at 37°.

free isoniazid unless the hydrazones were 50–100% broken down.

An attempt was made to see whether a hydrazone was stable inside the cell. An amount (1 g. dry wt.) of B.C.G. was suspended in 0·05% Tween 80 and incubated with 0·01 M-pyridoxal *iso*nicotinoylhydrazone for 24 hr. at 37°. After thorough washing and resuspension in water the cells were ruptured at 115° for 2 min. The resulting aqueous extract was separated by centrifuging and extracted with ethyl acetate in which the hydrazone would be more soluble than free isoniazid. The remaining aqueous extract and the ethyl acetate extract were concentrated to approx. 0·2 ml. Each was tested for free isoniazid before and after acid hydrolysis. Free isoniazid was detected only in the ethyl acetate extract after hydrolysis. The experiment therefore produced no evidence of the release of free isoniazid in the cell. It is important to the understanding of the mode of action of isoniazid that the behaviour of these hydrazones be studied further. They are inhibitory to the growth of *Mycobacterium*, and strains which are resistant to isoniazid are also resistant to the hydrazones. As the hydrazones cannot combine with pyridoxal phosphate they appear to discount the suggestion that the chemical inactivation of enzymes requiring pyridoxal phosphate explains the mode of action of isoniazid.

Transaminase activity in cells after long exposure to isoniazid

The evidence so far obtained indicated that transaminase inhibition could not explain the specific action of isoniazid. In order to test the effect of isoniazid on transaminases after incubation under conditions known to be bactericidal, cultures in Sauton medium were incubated with 100 μg. of isoniazid/ml. for 2- to 7-day periods. The isoniazid was added after the normal incubation period of 7 days at 37°. The extracts obtained from these cells still showed the aspartic–glutamic and isoleucine–glutamic acid transaminase reactions with rates comparable with extracts of untreated cells. No additions of pyridoxal phosphate were made to these extracts.

DISCUSSION

Reference has been made to Davison's (1956) experiments with isoniazid and pyridoxal phosphate. He observed that the rate of combination of isoniazid and pyridoxal phosphate was similar to the rate of inhibition of enzymes requiring pyridoxal phosphate, and suggested that the rate of inhibition by isoniazid might be used to demonstrate the requirement for pyridoxal phosphate. Davison did not record whether the rate of inhibition was affected by the substrate concentration. By this criterion the glutamic acid decarboxylase of *Proteus mirabilis* would not require pyridoxal phosphate. It would be of interest to know what the effect of isoniazid would be on an enzyme in which the pyridoxal phosphate was known to be firmly bound to the apoenzyme, for example the histidine decarboxylase described by Guirard & Snell (1954). The varying inhibition with the transaminases of B.C.G. and the insensitivity of the *Proteus* decarboxylase suggest that the inhibition by isoniazid may be determined by the ease with which the coenzyme may be removed from the apoenzyme.

Davison showed that the reaction between isoniazid and pyridoxal phosphate was rapid but the bactericidal effect of isoniazid is slow. Further, if Davison is correct in suggesting that all enzymes requiring pyridoxal phosphate are inhibited at the same rate, it would be necessary to postulate that the inhibition of all such enzymes resulted in the slow development of conditions intolerable to the organisms.

The evidence against transaminase as the site of action of isoniazid against *Mycobacterium* was that the transaminase activity remained after incubation with isoniazid under conditions known to be bactericidal and that transaminase activity was not inhibited by stable hydrazones of isoniazid which were known to have similar tuberculostatic activity to isoniazid. As far as could be determined, these compounds were stable in the presence of B.C.G. and the activity could not be attributed to the release of free isoniazid in the medium.

In the cell it is possible to visualize the equilibrium equation below, where X is the sensitive enzyme and INH is isoniazid.

$$\text{X} + \text{pyridoxal INH hydrazone} \rightleftharpoons \text{pyridoxal} + \text{INH} + \text{X} \rightleftharpoons \text{pyridoxal} + \text{INH–X}.$$

In such a system it is not possible to equate X with the transaminase system since this was not inhibited either by the pyridoxal *iso*nicotinoylhydrazone or by equimolar mixtures of isoniazid and pyridoxal.

SUMMARY

1. No qualitative differences in the range of transaminase activities of sensitive and resistant strains of *Mycobacterium tuberculosis* (B.C.G.) were found. The enzymes in extracts of sensitive and resistant organisms were equally sensitive to isoniazid.

2. Below a concentration of 0·05 M the amount of α-oxoglutarate employed in estimating the aspartic–glutamic transaminase activity affected the rate of the reaction and the degree of inhibition by a given concentration of isoniazid.

3. The presence of equimolar concentrations of pyridoxal phosphate prevented the inhibition by isoniazid. Stable hydrazones of isoniazid did not inhibit transaminase activity. These compounds, which were stable in the presence of heavy suspensions of B.C.G. organisms, had tuberculostatic activity comparable with isoniazid.

4. Transaminase activity was found in extracts of cells which had been exposed to isoniazid under bactericidal conditions.

5. It appears improbable that the bactericidal activity of isoniazid can be explained by its action on a transaminase or other enzyme requiring pyridoxal phosphate.

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Determination of Iron in Biological Material by Spectrophotometry of Ferric Perchlorate

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The determination of iron in inorganic samples by conversion into ferric sulphate or ferric perchlorate has been described by Bastian, Weberling & Pallila (1953, 1956). These salts absorb strongly at a region in the ultraviolet where interference from other elements is negligible. It therefore seemed likely that iron in biological material could be estimated by digestion with perchloric acid followed by spectrophotometry of the diluted digest. An accurate and convenient method results; as described, samples should contain 0·2–1 mg. of iron, but much smaller quantities of iron can be estimated by modification of the volumes. For convenience, the present procedure yields 100 ml. of solution for the optical measurement. It is, however, quite practicable to finish with a final volume of 10 ml. With this modification, iron can be estimated in the range 0·02–0·1 mg.

METHOD

Reagents. AnalaR perchloric acid (60%; sp.gr. 1·54) is taken to be 10N. By tenfold dilution of the concentrated acid, N-perchloric acid is prepared. AnalaR concentrated nitric acid is used. All reagents must be iron-free. Several batches of AnalaR acids have been used; they have all given acceptably low blank values.

Samples. An appropriate amount of material containing up to 1 mg. of iron is weighed or measured into a 250 ml. conical flask. For blood, 2 ml. is taken; for tissue such as liver or spleen, 2 g. of finely chopped material is weighed out; for urine, quantities varying from 5 to 50 ml. or more are necessary.

In the special case of serum-iron estimation, this method does not present any advantage over existing procedures. This is because the concentration of iron in the serum is exceedingly low and the method chosen must therefore be very sensitive. In addition, preliminary treatment of the serum with a precipitant of acid protein is necessary to remove traces of haemoglobin; the resulting filtrate is suitable for immediate development of colour with one of the conventional reagents.

Digestion. Nitric acid (5 ml.) is added to the contents of the flask and the mixture is boiled gently on a hot-plate for at least 1 hr., or longer if necessary, until a brown or yellow mobile liquid results. Further additions of nitric acid may be necessary to prevent drying. To the cooled solution 5 ml. of 10N-perchloric acid is added and the flask is returned to the hot-plate. The temperature is raised and the contents are allowed to evaporate to dryness. The heating should be adjusted so that 1½–2 hr. elapse between the addition of the perchloric acid and complete dryness. The residue is treated with 10 ml. of 10N-perchloric acid, which is heated until boiling to achieve complete solution of the residue. The cooled solution is washed quantitatively into a 100 ml. volumetric flask and

Table 1. *Comparison of iron determinations by different methods*

Sample	$TiCl_3$ titration (mg./100 ml.)	Spectrophotometry as cyanmethaemoglobin (mg./100 ml.)	Perchloric acid digestion (mg./100 ml.)
Blood	57·6	57·4	57·8
Blood	46·8	46·7	47·5
Blood	40·4	41·5	40·6
Blood	41·5	41·5	42·1
Blood	41·6	42·1	41·4
Blood	42·4	42·4	42·4
Blood	43·4	43·7	44·5
Blood	41·4	42·1	41·5
Blood	48·7	49·1	50·7
Blood	44·1	44·5	46·4
Liver	16·0*	—	20·0*
Spleen	114·0*	—	116·0*
Urine (cardiac failure)		—	1·3
Urine (haemoglobinuria in remission)		—	0·63
Urine (active haemoglobinuria)		—	12·2

* mg./100 g. wet wt.

made to the mark with water. If there is a considerable amount of potassium present in the residue, a crystalline precipitate of potassium perchlorate will form (this happens commonly with urine samples). It is readily removed by centrifuging the solution, and recovery experiments have shown that no iron is retained in the precipitate.

Standard. A permanent solution is prepared by dissolving 500 mg. of pure iron (Johnson, Matthey and Co. Ltd.) in a mixture of 60 ml. of nitric acid and 100 ml. of water, then diluting the solution to 1 l. A volume (2 ml.) of this stock standard (1 mg. of iron) is pipetted into a flask, 5 ml. of 10 N-perchloric acid is added and the mixture is evaporated to dryness. The residue is dissolved in 10 ml. of 10 N-perchloric acid and the solution made to 100 ml. with water. A simple solution of iron metal in dilute perchloric acid is unsuitable as a standard because the resulting ferrous perchlorate does not have the required ultraviolet absorption.

Blank. The blank used is N-perchloric acid. If, however, nitric acid of satisfactory purity is not available, it will be necessary to prepare a blank solution by evaporating to dryness a quantity of nitric acid equal to that used in the digestion, followed by treatment of the residue as specified above.

Spectrophotometry. The apparatus used was a Unicam quartz spectrophotometer SP. 500 fitted with a hydrogen-discharge lamp. Test and standard solutions are measured in 1 cm. silica cuvettes at 240 mμ. The instrument is set to 100% transmission with a cuvette filled with blank solution. The optical density is strictly proportional to the iron concentration up to a density of 0·8 at least, corresponding to 1 mg. of iron/100 ml.

Bastian *et al.* (1953) have emphasized the effect of temperature on the optical density. In the present method, measurements are made at room temperature (about 18°) and all solutions are allowed to stand for about 0·5 hr. before measurement to attain the same temperature.

RESULTS

The values obtained on a series of blood samples are given in Table 1, and compared with the precise but more exacting titration method of King & Wootton (1956). The table also shows the results obtained by spectrophotometry of cyanmethaemoglobin, a method which has been shown to give results in close agreement with the total blood iron. Comparative results on a sample of liver and spleen are recorded by the first two methods, although, for liver, the amount of iron in the sample was insufficient to allow an accurate titration. Some typical values of urine iron are shown in the table but no comparative values by other methods are included since there does not appear to be any satisfactory alternative procedure.

Table 2. *Optical density of various cations, after digestion with perchloric acid, at* mM *concentration and* 1 *cm. path length*

	240 mμ	260 mμ
Iron	4·2	2·9
Sodium	0·002	0·002
Potassium	0·004	0·004
Calcium	0·018	0·013
Magnesium	0·000	0·001
Copper	0·098	0·025

As a test of the precision of the method, a large sample of blood has been estimated on three separate occasions; each time six replicate portions of 2 ml. were digested. Measurements were made at 240 and 260 mμ. The mean value obtained for the iron content of the blood was 67·6 mg./100 ml., with the coefficient of variation of a single estimate equal to 2·4% at 240 mμ and 2·7% at 260 mμ.

Interference from the presence of other cations is, in general, negligible. Vanadium and molybdenum have an absorption comparable with that of iron; of the other elements only copper is likely to interfere, at least in animal specimens. If the sample

contains equimolar quantities of iron and copper, the iron result will be almost 2% too high, but, as shown by Bastian *et al.* (1956), this interference can be reduced by measurement at 260 mμ instead of 240 mμ, with some sacrifice of sensitivity. The effect of other common cations is given in Table 2.

DISCUSSION

Spectrophotometry of ferric perchlorate has proved to be a quick and simple method for analysis of total iron. The determination can be completed during the course of one day and the total working time is small. It is possible to shorten the method a little by measuring the optical density of the diluted perchloric acid digest (without taking to dryness), but it is essential to remove the last traces of nitric acid, and it is therefore convenient to evaporate the digest completely and redissolve the inorganic material in a fresh portion of perchloric acid. Recovery experiments have shown that no iron is lost even when the dry residue is heated for several hours.

Digestions with perchloric acid have a bad reputation for explosions. In the present method, organic matter receives a thorough treatment with boiling concentrated nitric acid before any perchloric acid is added. We have carried out many digestions in this way without incident. In the final stages a solution of inorganic salts in perchloric acid is evaporated to dryness; in the absence of organic matter this process is acceptable as safe. However, in view of the consequences of mismanagement, all digestions are conducted behind a plastic screen and precautions are taken to avoid contact of hot liquid or vapour with inflammable materials.

SUMMARY

A rapid and precise method for determining iron in biological samples is described. Iron in the range 0·02–1 mg. is estimated by digestion with nitric acid–perchloric acid and ultraviolet spectrophotometry at 240 mμ. Precautions, and the effect of other cations, are discussed.

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Studies in Detoxication

73. THE METABOLISM OF ALKYLBENZENES: PHENYLACETYLENE AND PHENYLETHYLENE (STYRENE)*

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In the previous papers in this series (Smith, Smithies & Williams, 1954*a–c*; Robinson, Smith & Williams, 1955; Robinson & Williams, 1955; El Masri, Smith & Williams, 1956), the metabolism of alkylbenzenes containing saturated side chains was investigated. These studies have now been extended to include the unsaturated derivatives styrene and phenylacetylene. Of these compounds styrene, a compound of considerable importance in the plastics and other industries, has been previously investigated. In man, rats and rabbits, it appears to be metabolized largely by loss of one of its side-chain carbon atoms to yield benzoic acid, which is excreted as hippuric acid (Spencer, Irish, Adams & Rowe, 1942; Carpenter, Shaffer, Weil & Smyth, 1944), and in rats and rabbits it is reported that 50–90% of the dose may be excreted as hippuric acid. Recently, however, Danishefsky & Willhite (1954) reported briefly some experiments using [β-^{14}C]styrene and found that, when styrene was injected subcutaneously into rats (dose about 0·1 ml./100 g. rat), about 12% of the radioactivity appeared in the expired air as carbon dioxide whereas over 70% appeared in the urine. Spencer *et al.* (1942) suggested that a small amount of a mercapturic acid was formed, but, apart from hippuric acid, small amounts of unchanged styrene and respiratory CO_2, the metabolites of styrene have not been identified. Phenylacetylene has not been investigated hitherto.

* Part 72: Mead, Smith & Williams (1958).

EXPERIMENTAL

Materials and Methods

Animals. Rabbits kept on a constant diet of 50 g./day of rat cubes (Diet 41, Associated Flour Millers) were used.

Reference compounds. Phenylacetylene (b.p. 143°/760 mm.) and styrene (b.p. 58°/20 mm., n_D^{23} 1·545) were redistilled immediately before use. (−)-Mandelic acid [m.p. 133°; $[\alpha]_D^{25}$ −154° in water (*c*, 1)] was converted into S-*benzylthiuronium* (−)-*mandelate* which formed plates from water, m.p. 180° and $[\alpha]_D^{25}$ −57° in 20% (v/v) aqueous ethanol (*c*, 1) (Found: C, 60·6; H, 5·9. $C_{16}H_{18}O_3N_2S$ requires C, 60·4; H, 5·7%) [the (±)-mandelate is a known compound, m.p. 166°; see Wild (1947)]. (±)-Phenylglycol, m.p. 67°, was prepared by hydrolysis of styrene epoxide with water acidified with a few drops of $HClO_4$. Phenacyl alcohol, m.p. 73°, was prepared by boiling phenacyl bromide with barium carbonate for 12 hr. The properties of these compounds agreed with those found in the literature.

Analytical methods. Glucuronic acid in urine was determined according to Paul (1951) (see Mead *et al.* 1958), ethereal sulphates according to Sperber (1948), hippuric acid according to El Masri *et al.* (1956) and mercapturic acid by iodine titration according to Stekol (1936). Styrene and phenylacetylene did not interfere with the mercapturic acid estimations.

Phenylacetylene or styrene in expired air was collected and determined spectrophotometrically by using the apparatus described by Parke & Williams (1950; see also Azouz, Parke & Williams, 1952). The expired air of the animals dosed with phenylacetylene or styrene was dried over anhydrous $MgClO_4$ and the hydrocarbons present were then trapped in ethanol contained in wash bottles cooled in acetone/solid CO_2. The phenylacetylene or styrene content of the ethanol was estimated at intervals by measuring the light-absorption at 235 mμ for phenylacetylene (the $\epsilon_{max.}$ found at this wavelength for pure phenylacetylene was 16 000), and at 245 mμ for styrene ($\epsilon_{max.}$ found was 14 100), and identity of the material analysed was checked by determining the absorption curve over the range 220–270 mμ with a Unicam SP. 500 spectrophotometer (for published spectra see Magat & Maier, 1943). Quantities of 0·5 g. of phenylacetylene on a watch glass placed in the tank were recovered quantitatively in 12 hr. by aeration into the ethanol absorption bottles. With styrene in similar quantities, the maximum recovery in two experiments was 89·1 and 84·2% after 12 hr. aeration. Recovery was incomplete because styrene tends to polymerize, and in these experiments 2·2 and 2·7% of the original styrene remained on the watch glass as polymeric material.

Paper chromatography. The separation and identification on paper of hippuric, phenaceturic and *p*-hydroxyphenylacetic acid in urine were carried out as described by El Masri *et al.* (1956). Rough estimates of phenaceturic acid, which is the only metabolite of phenylacetylene, were obtained by comparing the spots from the unknown material with spots from known amounts of the acid added to urine. Mandelic acid, phenylglycol and phenylglycol glucuronide (about 50 μg.) were detected chromatographically on paper; the solvent systems, R_F values and colour reactions are quoted in Table 1.

Table 1. *R_F values and colour reactions for the detection of the metabolites of styrene*

Solvent systems used were: *A*, benzene–acetic acid–water (1:1:2, by vol.) with Whatman no. 1 paper; *B*, ethyl methyl ketone–2N-ammonia (1:1) with Whatman no. 1 paper; *C*, butanol–acetic acid–water (4:1:5, by vol.) on Whatman no. 4 paper; and *D*, propanol–aq. ammonia (sp.gr. 0·88) (7:3, v/v) with a Whatman no. 4. All the descending chromatograms were run for 5 hr. For the detection of mandelic acid and phenylglycol, the paper was soaked on a glass plate with a mixture of concentrated sulphuric acid (20 ml.) and 40% (w/v) formalin (0·2 ml.), which showed up these compounds as brown spots. Hippuric and phenaceturic acid gave no colours with this reagent.

	R_F values in solvent			
	A	*B*	*C*	*D*
Mandelic acid	0·13	0·13	0·90	0·55
Phenylglycol	0·22	0·71	0·93	0·86
Phenylglycol glucuronide	0·00	0·00	0·55	0·26
Hippuric acid	0·07	0·07	0·86	0·55

Isolation of metabolites from phenylacetylene

The urine from rabbits receiving oral doses of phenylacetylene (0·4 g./kg.) was non-reducing. It contained no ketones when tested with 2:4-dinitrophenylhydrazine and no glucuronides when tested with naphtharesorcinol. Quantitative measurements showed no significant increase above normal in the glucuronic acid or ethereal sulphate outputs. Paper chromatography of the urine indicated that phenaceturic acid was being excreted in small amounts for at least 4 days after dosing, and by comparison with reference chromatograms it was estimated that 5–15% of the dose of phenylacetylene was being excreted daily as phenaceturic acid for 3 days after dosing. No significant increase in hippuric or *p*-hydroxyphenylacetic acid was found. Mandelic acid was not detected in the urine by

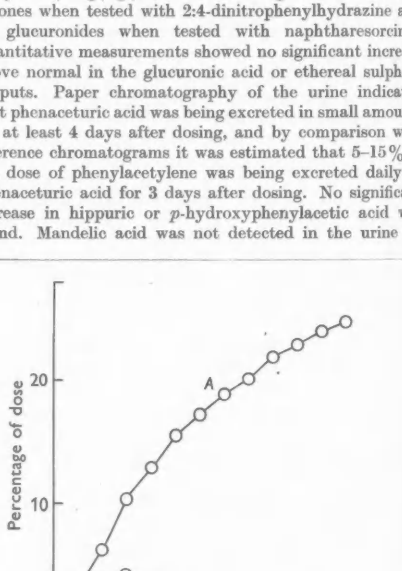


Fig. 1. Elimination of phenylacetylene in the expired air of a rabbit which had received a 1·5 g. oral dose of the hydrocarbon. Curve *A* shows the total phenylacetylene eliminated and curve *B* the amount eliminated every 6 hr.

direct paper chromatography, but traces of it were detected by its R_F values in four solvents (see above) in concentrated extracts of the acidified urine.

Isolation of phenaceturic acid. A rabbit was given 2 g. of phenylacetylene by stomach tube and urine collected daily for 4 days. Each day's urine was adjusted to pH 2 by the addition of conc. HCl and continuously extracted with ether for 18 hr. No ketones were detected in the extracts. On removal of the ether each extract left a tarry residue which crystallized on adding 5 ml. of water. The first and second days' urine each yielded 0·1 g. of phenaceturic acid, m.p. and mixed m.p. 142°, and the combined extracts from the third and fourth days' urine gave another 0·1 g. (total, 7·5% of the dose).

Elimination of unchanged phenylacetylene. Two experiments were made in which the expired air of rabbits which had received 1·5 g. of phenylacetylene orally was collected and analysed. In the first experiment 25% and in the second 37% of the dose was found in the expired air as unchanged phenylacetylene during 3 days (72 hr.) after dosing. At the end of 3 days phenylacetylene was still being eliminated in the expired air. The slow rate of elimination of this hydrocarbon is shown in Fig. 1.

Isolation of metabolites from styrene

The urine of rabbits which had received styrene (0·5 g./kg.) orally gave a weak naphtharesorcinol reaction, but was non-reducing and gave no reaction for ketones. Paper chromatography of the urine showed the presence of large amounts of hippuric acid, but no significant amounts of phenaceturic acid. Mandelic acid was readily detected, together with an unidentified product which gave a pink colour with the sulphuric acid–formalin reagent (see Table 1).

Isolation of (+)-mandelic acid. The 24 hr. urine of eight rabbits which had received collectively 16 g. of styrene was acidified with 10N-HCl to a pH of about 2 and then continuously extracted (24 hr.) with $CHCl_3$. The latter on evaporation left a dark-brown mixture of tar and crystals which was stirred with cold water (20 ml.) and filtered. After being kept overnight the crystals were collected and identified as hippuric acid (m.p. and mixed m.p. 189°). The filtrate from the crystals was neutralized and treated with benzylthiuronium chloride (see Wild, 1947) when a precipitate (0·55 g.) of *S-benzylthiuronium (+)-mandelate* separated. On recrystallization from water, this salt formed colourless plates, m.p. 176° and $[\alpha]_D^{25}+57°$ in 20% (v/v) aqueous ethanol (*c*, 1) (Found: C, 60·1; H, 5·6; N, 8·6. $C_{16}H_{18}O_3N_2S$ requires C, 60·4; H, 5·7; N, 8·8%). This salt was similar to the corresponding salt of (−)-mandelic acid already prepared (see above) but had the opposite sign of rotation.

Isolation of a glucuronide. The urine from twelve rabbits which had been fed with a total of 22 g. of styrene was fractionated with lead acetate (Kamil, Smith & Williams, 1951). The aqueous glucuronide fraction was separated from the basic lead acetate precipitate and concentrated *in vacuo* to small bulk. The concentrated solution which had a positive rotation was extracted with $CHCl_3$ (3 × 500 ml.) to remove mandelic acid, which was eventually isolated as *S*-benzylthiuronium (+)-mandelate (1·5 g.), m.p. 176° and $[\alpha]_D^{25}+56°$ (in 20%, v/v, aqueous ethanol). The aqueous residue, which had now a small negative rotation, was evaporated *in vacuo* to a gum. The latter was dissolved in a little methanol and methylated with diazomethane, and the product was acetylated with acetic anhydride and pyridine to form the acetylated methyl ester of the glucuronide present. This was obtained as a partly crystalline solid on pouring the acetylation mixture into an excess of water. This solid was purified by solution in $CHCl_3$, drying over anhydrous $CaCl_2$, filtration and evaporation of the solvent to leave again a partly crystalline solid. It was now heated to 160° at 0·5 mm. pressure to distil off a few drops of an unidentified oil (possibly methyl mandelate). The residue now readily crystallized (0·1 g.) on rubbing with ethanol. Repeated recrystallization with ethanol yielded colourless needles (55 mg.) of *methyl (phenylglycol monoacetate tri-O-acetyl-β-D-glucosid)uronate*, which melted at 140° then solidified and melted again at 155°, and showed $[\alpha]_D^{25}+1\pm2°$ in $CHCl_3$ (*c*, 1) (Found: C, 55·9; H, 5·7; OMe, 6·1%. $C_{23}H_{28}O_{12}$ requires C, 55·7; H, 5·7; OMe, 6·3%). This ester gave an intense naphtharesorcinol reaction and was identical with the glucuronide prepared by feeding phenylglycol (see below). The presence of phenylglycol in the glucuronide was proved by hydrolysing the ester with N-NaOH at 60° for 0·5 min., then treating the solution with a preparation of snail-gastric-juice glucuronidase at pH 4·5 (Britton and Robinson buffer mixture; British Drug Houses Ltd.). The enzymic hydrolysate was extracted with ether and the presence of phenylglycol in the extract was shown by paper chromatography with the three solvent systems already described for the identification of this glycol (see Table 1).

Elimination of unchanged styrene. In two experiments carried out as with phenylacetylene, 2·1 and 3% of doses of 1·8 g. of styrene were recovered in the expired air.

Isolation of metabolites from phenylglycol

The metabolism of phenylglycol was investigated because this glycol was a metabolite of styrene. A qualitative examination of the urine of rabbits given orally 0·8 g./kg. of (±)-phenylglycol showed that its metabolites were hippuric acid, mandelic acid and a glucuronide, the first two being readily detected by paper chromatography.

The basic lead acetate fraction of the 24 hr. urine of ten rabbits dosed with a total of 20 g. of (±)-phenylglycol was prepared in the usual manner and freed from lead. The lead-free filtrate was concentrated to 20 ml. and the hippuric acid and mandelic acid removed with $CHCl_3$ (4 × 500 ml.) as described above. Evaporation of the chloroform extract and solution of the residue in water (10 ml.) yielded a small amount of hippuric acid (m.p. and mixed m.p. 189°) and treatment of the residue with benzylthiuronium chloride yielded 5·1 g. of *S*-benzylthiuronium mandelate, which appeared to be mainly the (+)-isomer ($[\alpha]_D^{25}+36°$, in 20% aqueous ethanol; *c*, 1). The aqueous solution after extraction with $CHCl_3$ was evaporated to dryness, and the residue methylated with diazomethane and acetylated with pyridine and acetic anhydride. The acetylated methyl ester of the glucuronide was purified exactly as described in the section on styrene. Methyl (phenylglycol monoacetate tri-*O*-acetyl glucosid)uronate (120 mg.) was obtained as long needles again showing two melting points, 140° and 155°, ($[\alpha]_D^{25}+1°$ in $CHCl_3$) (Found: $CH_3{\cdot}CO$, 34·2. $C_{22}H_{28}O_{12}$ requires $CH_3{\cdot}CO$, 34·7%), and it did not depress the melting points of the compound obtained from styrene. That it contained phenylglycol was

again proved by deacetylating and demethylating, hydrolysing the product with snail glucuronidase, and separating the phenylglycol by paper chromatography.

Styrene oxide. Smith *et al.* (1954*b*) had previously shown that this epoxide when fed to rabbits yielded conjugated glucuronic acid corresponding to about 15% of the dose (0·4 g./kg.), but they were unable to isolate and identify the glucuronide. We have now found that hippuric acid (9–10% of the dose, see Table 2) and mandelic acid are also metabolites of this compound.

A sample (0·05 ml.) of the urine of a rabbit which had received 1·6 g. of styrene epoxide was chromatographed in the four solvent systems *A*, *B*, *C* and *D* (see Table 1). Hippuric acid was readily detected in amounts of the order of 10% of the dose; mandelic acid was also found in amounts of the same order. However, neither phenylglycol nor its glucuronide could be detected.

Methylphenylcarbinol. The main metabolites of this compound are already known (Smith *et al.* 1954*b*). The urine of a rabbit which had received 2 g. of this compound orally was chromatographed as above, but neither phenylglycol nor its glucuronide was found, although small amounts of mandelic acid and very large amounts of a glucuronide (probably methylphenylcarbinol glucuronide) were detected.

Phenacyl alcohol ($C_6H_5 \cdot CO \cdot CH_2 \cdot OH$). Quantitative determinations showed that when phenacyl alcohol (0·8 g./kg.) was fed to rabbits the normal ethereal sulphate and hippuric acid outputs were unaltered, but the glucuronides rose slightly to not more than about 5% of the dose. The main object in feeding phenacyl alcohol was to find out whether it gave a glucuronide of (+)- or (−)-phenylglycol, but neither phenylglycol nor its metabolite, mandelic acid, was excreted. A glucuronide, however, was isolated. Two rabbits were each given 1·9 g. of phenacyl alcohol by stomach tube. The 24 hr. urine (100 ml.) gave a positive naphtharesorcinol reaction, a small precipitate with 2:4-dinitrophenylhydrazine in HCl, but a negative test for reducing sugars. This urine was fractionated by the lead acetate procedure. Neither the normal nor basic lead acetate fraction contained any mandelic acid. The basic lead acetate fraction, however, contained a small amount of a glucuronide. The lead was removed from this fraction and the resulting aqueous solution was evaporated to dryness. The residue was dissolved in water (100 ml.), filtered and treated with an excess of Brady's reagent (2:4-dinitrophenylhydrazine in dilute HCl). A red microcrystalline precipitate (120 mg.) was formed which, on recrystallization from ethanol containing a trace of HCl, yielded fine red needles, m.p. 225° (decomp.) and $[\alpha]_D^{25} - 154°$ in pyridine (*c*, 0·8). This substance analysed as the 2:4-*dinitrophenylhydrazone* of *benzoylmethyl glucosiduronic acid* (phenacyl alcohol glucuronide) (Found: C, 48·2; H, 4·4; N, 11·0. $C_{20}H_{20}O_{11}N_4$ requires C, 48·8; H, 4·1; N, 11·4%). Phenylglycol glucuronide was not found and the metabolism of phenacyl alcohol was not further investigated.

RESULTS AND DISCUSSION

A summary of the quantitative results obtained is given in Table 2.

Phenylacetylene. The metabolism of phenylacetylene in the rabbit appears to be a relatively simple process and the formation of its single metabolite can be explained by the simple addition of water to the triple bond. The compound, however, appears to be very slowly metabolized since unchanged phenylacetylene (see Fig. 1) and its urinary metabolite, phenaceturic acid, are eliminated for at least 4 days after dosing, and it appears that approximately half of the dose (0·4 g./kg.) is eliminated unchanged and the other half converted into phenylacetic acid. The addition of water to the triple bond appears to be asymmetric, since the formation of phenylacetic acid requires that a hydroxyl group be added to the β- and a hydrogen atom to the α-carbon atom, thus:

$$\mathrm{Ph \cdot C \vdots CH} \xrightarrow{+H_2O} \mathrm{Ph \cdot CH{:}CH \cdot OH} \rightleftharpoons \mathrm{Ph \cdot CH_2 \cdot CHO} \xrightarrow{\text{oxidation}} \mathrm{Ph \cdot CH_2 \cdot CO_2H}.$$

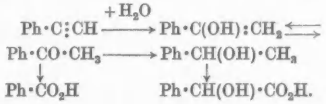
Table 2. *Quantitative aspects of the metabolism of phenylacetylene, styrene and phenylglycol in the rabbit*

The figures are the average for three animals, the ranges being given in parentheses.

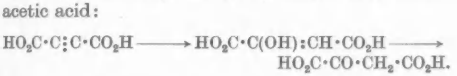
	Dose (m-moles/kg.)	Phenylacetylene* 4	Styrene† 5	Styrene† epoxide 4	Phenylglycol† 3
Percentage of dose eliminated as	Unchanged in expired air	31 (25, 37)[2]	2 (2, 3)[2]	—	—
	Glucuronide	0 (0–1)	6 (4–7)	15‡	34 (20–29)
	Ethereal sulphate	0 (0–0)	0 (0–0)	0‡	0 (0–0)
	Hippuric acid	0§	40 (37–44)	9·5 (9–10)	7 (4–13)
	Phenaceturic acid	About 30§	0§	0§	0§
	Mandelic acid	Trace	+ + (2–3% by isolation)	About 10%‖	+ + + (11% by isolation)‖
	Mercapturic acid	0 (0–0)¶	5 (2–7)¶	—	—

* These figures are for 3 days after dosing, after which time the excretion of metabolites was still incomplete.
† Here excretion of metabolites was complete in 1–2 days.
‡ Values taken from Smith *et al.* (1954*b*).
§ As shown by paper chromatography.
‖ Mandelic acid was a significant metabolite of these compounds, but we did not find a quantitative method which gave satisfactory results.
¶ As estimated by the iodometric method of Stekol (1936).

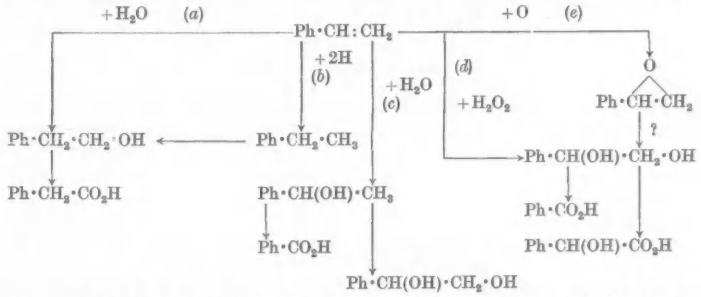
If the addition occurs in the reverse manner, then acetophenone and its metabolites—methylphenylcarbinol, hippuric acid and mandelic acid—would be expected to be formed (Smith *et al.* 1954*b*) and excreted thus:



Except for traces of mandelic acid, none of these metabolites was found and therefore it can be concluded that the hydration of the triple bond is asymmetrical. The view that the triple bond can be hydrated in biological systems is supported by the observation of Eimhjellen (1956) that cell-free extracts of a strain of bacteria from soil and sewage (Enterobacteriaceae) can convert acetylenedicarboxylic acid by direct hydration into oxaloacetic acid:



Styrene. The metabolism of styrene is more complicated than that of phenylacetylene. The metabolites which we have to account for are hippuric acid, phenylglycol and mandelic acid. The following routes of metabolism might be expected:



Routes (*a*) and (*b*) can be eliminated because it is known that phenylethanol (*a*) and ethylbenzene (*b*) give rise to phenaceturic acid (Smith *et al.* 1954*b*; El Masri *et al.* 1956) which is not a metabolite of styrene. Routes (*c*) and (*e*) are possible since both methylphenylcarbinol (*c*) and styrene epoxide (*e*) yield hippuric acid and mandelic acid in the urine, but neither of them gives rise to phenylglycol, although this compound could be an intermediate in their metabolism without appearing in the urine. Route (*d*) is a 'perhydroxylation', a reaction which has been postulated for the formation of dihydrodiols during the metabolism of aromatic hydrocarbons. If this process is postulated in the metabolism of styrene it would account for the formation of all three of its metabolites.

Two further comments appear to be necessary: the first concerns the monoglucuronide of phenylglycol, of which four isomers are possible. This glucuronide could be a 1- or 2-glucuronide of the (+)- or (−)-glycol [(+)- or (−)-Ph·CH(OG)·CH_2OH or (+)- or (−)-Ph·CH(OH)·CH_2·OG, where G is $-C_6H_9O_6$]. At present we have no data to decide which isomer we isolated. The second concerns the optical nature of the mandelic acid isolated as metabolite of styrene. The isomer isolated was the (+)-form and this suggests that the double bond of styrene is saturated in such a way as to produce an asymmetric α-carbon atom in the metabolites.

SUMMARY

1. The metabolic fate of phenylethylene (styrene) and phenylacetylene in the rabbit has been investigated.

2. Phenylacetylene is slowly metabolized to phenaceturic acid, which is excreted in the urine for several days after a single oral dose (0·4 g./kg.). Roughly 30–40 % of the dose is eliminated in the unchanged state in the expired air over 3 days, and about the same amount is excreted in the urine as phenaceturic acid. It is suggested that phenylacetylene is asymmetrically hydrated *in vivo* to the enol form of phenacetaldehyde.

3. Styrene does not yield phenaceturic acid *in vivo*. 'Its main metabolite is hippuric acid (30–40 % of the dose). It also forms mandelic acid and phenylglycol, the latter being excreted as a monoglucuronide. Only about 2 % of the styrene administered is eliminated unchanged in the expired air.

4. (±)-Phenylglycol when fed to rabbits also yields hippuric acid, mandelic acid and phenylglycol glucuronide, and it appears to be an intermediate in the metabolism of styrene. It is suggested that styrene may undergo 'perhydroxylation' *in vivo* to phenylglycol.

5. Styrene epoxide and methylphenylcarbinol, which are possible intermediates in styrene metabolism, do not lead to the excretion of phenylglycol

when administered to rabbits, although they yield hippuric and mandelic acids.

6. Phenacyl alcohol is not reduced to phenylglycol in the rabbit. A small proportion (< 5 %) of it appears to be excreted as phenacyl alcohol glucuronide, which was isolated.

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The Preparation and Properties of Ergothioneine Disulphide

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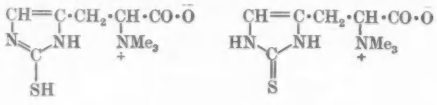
Ergothioneine occurs both intracellularly in blood (Hunter & Eagles, 1927) and extracellularly in seminal plasma (Mann & Leone, 1953), but it has never been shown to occur in either a combined form or in a higher state of oxidation.

Although ergothioneine is usually written in the thiol form (I) it is not a normal thiol compound as it does not give a nitroprusside reaction; neither, however, does it give the blue colour with the Grote reagent (Grote, 1931) typical of thiourea and substituted thioureas (Kjaer & Rubinstein, 1953) (II).



(I) (II)

The chemical reactions of the sulphur in the mercaptoglyoxalines are different from those of normal thiols, in that the sulphur is completely unaffected by boiling 50 % aqueous potassium hydroxide (Barger & Ewins, 1911), but is rapidly cleaved from the glyoxaline ring in the form of sulphate by even weak oxidants such as ferric sulphate (Ashley & Harington, 1930), to yield the corresponding glyoxaline. As a generalization it can be stated that the majority of the 2-mercaptoglyoxalines do not form stable disulphides, sulphenic or sulphinic acids, but this depends on the substituents in the 4 and 5 positions of the glyoxaline ring and there are a few exceptions to this rule. For a complete review of the chemistry of these compounds see Heath (1951). There is only one report of a higher oxidation product of ergothioneine (Barger & Ewins, 1911). By the action of iodine on ergothioneine they obtained black, steel-grey or blue, mixed crystals of a periodide which they stated was that of ergothioneine disulphide. No evidence or analysis in support of this statement was given; it is probable that the product, besides being a periodide, is also iodinated in the 4(5) position, since this halogen substitution readily occurs as was shown in the synthesis of 4-bromo-5-methyl-2-glyoxalyl disuphide (Heath, Lawson & Rimington, 1951).

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Circumstantial evidence, however, that ergothioneine might be catalytically oxidized is to be found in several early papers on the oxidation of cysteine and glutathione. Woodward & Fry (1932) showed that whereas a pure solution of ergothioneine is not oxidized by iodine, under their conditions for the estimation of glutathione, it is oxidized in the presence of glutathione. Pirie (1933) also showed from polarimetric observations that ergothioneine catalysed the hydrogen peroxide oxidation of cysteine.

The biochemical function of ergothioneine is not known, and, although from the organic chemical approach it seemed unlikely that ergothioneine disulphide could exist as a stable compound, we decided to investigate whether ergothioneine disulphide could exist even temporarily as a metabolic intermediary. Some of these results have been briefly reported elsewhere (Heath & Toennies, 1955).

EXPERIMENTAL

By analogy with the chemistry of the mercaptoglyoxalines as a whole, it was obvious that the mildest possible conditions of oxidation would be necessary for the formation of ergothioneine disulphide, and in the first instance the effect of oxygen at various pH levels and in the presence and absence of catalysts was studied. The reactions were followed spectrophotometrically by using a Beckman Model DU spectrophotometer.

The ultraviolet-absorption spectra of ergothioneine and thiourea, 50 μM in water, pH 7, are shown in Fig. 1. The change in wavelength of the maximum in the spectrum of ergothioneine with change of pH is shown in Fig. 2. The spectrum of ergothioneine at all pH values below 9 has a sharp maximum at 258 mμ, log E 4·16; as the pH is increased above 9 the maximum is displaced to shorter wavelengths and becomes progressively broader. The ultraviolet-absorption spectrum of thiourea (Mason, 1954) has a sharp maximum at 236 mμ, log E 4·08, and there is no change in the wavelength of the maximum between pH 2·4 and 12·1. There is no absorption in the spectrum of either compound above 300 mμ at any pH. The pH values of the solutions were determined with a Beckman pH meter. For the ultraviolet measurements corresponding solutions of the buffers were used as blanks.

Action of oxygen on ergothioneine in the presence of glutathione

The presence of an equimolar concentration of reduced glutathione does not affect the ultraviolet spectrum contributed by ergothioneine (50 μM) at wavelengths greater than 230 mμ.

A mixture of 0·01 M-ergothioneine hydrochloride (1 ml.) and 0·01 M-reduced glutathione (2 ml.) was slowly aerated in water for 1·5 hr. in a weighed apparatus; at the end of the aeration period, the solution was made up to the original weight with water and a portion was diluted for ultraviolet measurement. No change in the ultraviolet spectrum of ergothioneine was observed, and the solution still gave a strongly positive nitroprusside test for reduced

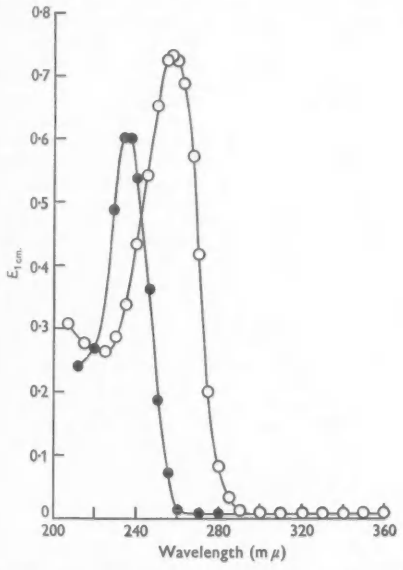

Fig. 1. Ultraviolet-absorption spectrum of (*a*) ergothioneine (○) and (*b*) thiourea (●) (50 μM in water); pH 7.

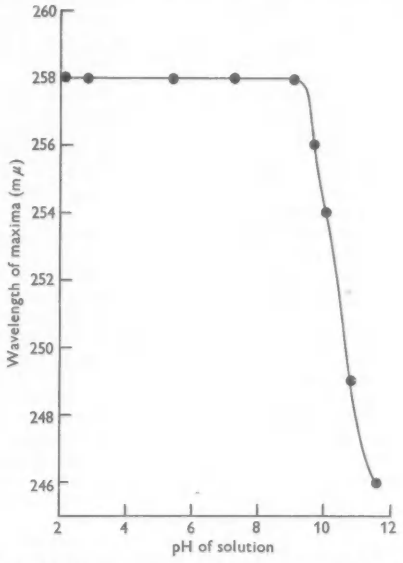

Fig. 2. Relationship between the wavelength (mμ) of the maximum in the ultraviolet-absorption spectrum of ergothioneine and pH.

glutathione. Hence little, if any, oxidation of either ergothioneine or glutathione had occurred.

As glutathione is not oxidized by atmospheric oxygen in the absence of heavy metals at low pH values, but is rapidly oxidized in the presence of copper, this rate increasing with increase in pH (Lyman & Barron, 1937), 5 ml. of 0·01 M-glutathione, 0·5 ml. of 0·001 M-$CuSO_4$ and 0·5 ml. of 10% sodium acetate–1% Na_2CO_3 soln. (pH 10·5) were mixed and aerated under the same conditions. After 1 hr. the solution failed to give the nitroprusside reaction, showing that complete oxidation of the glutathione had occurred. A solution (5 ml.) of 0·01 M-ergothioneine was then aerated in the same way, but after 2 hr. there was no change in the ultraviolet spectrum and oxidation of the ergothioneine had not occurred. On aeration of a mixture of 0·01 M-ergothioneine and glutathione under the same conditions, the glutathione was oxidized but the ergothioneine was not. After standing at room temperature for 60 hr. there was still no change in the ergothioneine spectrum. The solution was adjusted to pH 2 with HCl, and it was then observed that after standing for 24 hr. the ergothioneine ultraviolet maximum at 258 mμ was greatly diminished, although there was no appearance of reduced glutathione (GSH). It seemed therefore that at pH 2, in the presence of copper and oxidized glutathione (GSSG), ergothioneine (ESH) was oxidized but the reaction GSSG + ESH → GSSE + GSH did not take place as there was no corresponding formation of GSH. It was further demonstrated that a similar fall in the ultraviolet maximum was caused when ergothioneine was aerated at pH 2 in the presence of Cu^{2+} ions but in the absence of glutathione.

Effect of Cu^{2+} *ions, hydrochloric acid concentration and cysteine on the oxidation of ergothioneine*

A series of solutions of mM-ergothioneine, in water, 0·01 N-HCl and 0·1 N-HCl, both with and without 10^{-5} M-$CuSO_4$, were made in glass-stoppered tubes. For ultraviolet measurements 0·5 ml. of these solutions was diluted to 10 ml. with water and read against a corresponding blank solution. After the initial reading the solutions were saturated with oxygen and allowed to stand at room temperature. The ultraviolet spectra of fresh dilutions of the reaction mixtures were read daily for 8 days. Then a fresh dilution of 0·5 ml. of the oxidized ergothioneine solution was made with 9·5 ml. of mM-cysteine hydrochloride and the corresponding dilution of cysteine used as the blank in the ultraviolet-spectra measurements. The results of these ultraviolet measurements showed that ergothioneine is not oxidized at all by oxygen in pure aqueous solutions, but that a slow oxidation occurs in acid solution and in the absence of Cu^{2+} ions, as indicated by the change in the spectrum, which could be restored to the original ergothioneine spectrum by the addition of cysteine. The addition of copper to these solutions caused, on long standing, a change in spectrum which was not reversed by the addition of cysteine.

When the same experiment was carried out in 5 N-HCl, no detectable oxidation occurred after 2 hr. in the absence of Cu^{2+} ions, but in the presence of 10 μM-$CuSO_4$ a 20% reduction in the maxima at 258 mμ occurred and the change in spectrum after 24 hr. is shown in Fig. 3. Curve (*a*) is the ultraviolet spectrum of the oxidized ergothioneine. Curve (*b*) is this same oxidized product after treatment with cysteine; this spectrum is identical with the spectrum of ergothioneine. Thus ergothioneine is reversibly oxidizable. The oxidation of ergothioneine by aeration in 5 N-HCl in the presence of Cu^{2+} ions is slow and was not suitable as a preparative method; in one experiment it took 7 days' aeration to complete the oxidation of 100 mg. of ergothioneine when dissolved in 10 ml. of 5 N-HCl, mM in Cu^{2+} ions.

Hydrogen peroxide oxidation of ergothioneine

A series of solutions (4 ml.) of 5 mM-ergothioneine hydrochloride in water, 0·05 N, 0·5 N- and 5 N-HCl, both with and without 10 μM-$CuSO_4$, were made and to each, with rapid mixing, was added 0·4 ml. of 0·05 N-H_2O_2. After standing 30 min., 0·11 ml. of each solution was diluted to 10 ml. (*a*) with water, (*b*) with mM-cysteine hydrochloride, for ultraviolet measurement, and in each the corresponding blank solutions were used. The results given in Table 1 show only the absorption at 258 mμ. As oxidations occurred there was also a rise in the absorption at wavelengths greater than 290 mμ similar to that shown in Fig. 3 for the oxidation of ergothioneine with oxygen. The results after 30 min. show that the largest fall in absorptions at 258 mμ occurred in 5 N-HCl. The original spectrum of ergothioneine was restored by the addition of cysteine, in all cases excepting the reaction in water for 24 hr., which indicated that in the latter case some other non-reversible oxidation had occurred. The reactions were the same in the absence as in the presence of Cu^{2+} ions.

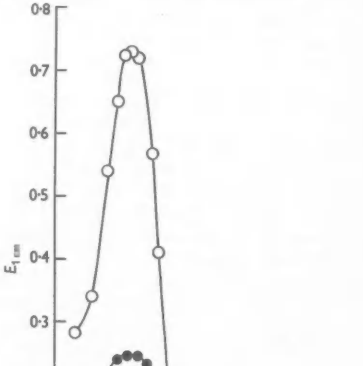

Fig. 3. Ultraviolet-absorption spectra of (*a*) ●, ergothioneine after oxidation with oxygen in 5 N-HCl containing 10 μM-$CuSO_4$, (*b*) ○, the same oxidized product after treatment with cysteine hydrochloride.

Table 1. *Hydrogen peroxide oxidation of ergothioneine in water, 0·05N-, 0·5N- and 5N-HCl in the presence and absence of Cu^{2+} ions and cysteine*

$E_{1\,cm.}$ of 50 μM-ergothioneine at 258 mμ after treatment with H_2O_2.

	After 30 min.		After 24 hr.	
	Cysteine absent	Cysteine present	Cysteine absent	Cysteine present
Water	0·540	0·740	0·397	0·653
Water + Cu^{2+} ions	0·510	0·730	0·404	0·665
0·05N-HCl	0·323	0·735	0·275	0·740
0·05N-HCl + Cu^{2+} ions	0·327	0·738	0·286	0·740
0·5N-HCl	0·267	0·732	0·265	0·732
0·5N-HCl + Cu^{2+} ions	0·256	0·740	0·250	0·735
5N-HCl	0·232	0·745	0·240	0·735
5N-HCl + Cu^{2+} ions	0·235	0·742	0·255	0·730
Control in water without peroxide	0·740	0·740	0·740	0·740

The formation of the disulphide (proof of the disulphide nature of the oxidation product will be given later) can be seen by the development of a yellow colour in the solution both in the oxygen and peroxide oxidations. This colour is formed immediately by peroxide in the 5N-HCl solution, but only develops later with the weaker acid concentration.

Paper chromatography

A systematic search for a satisfactory solvent system which could resolve ergothioneine, ergothioneine disulphide (ESSE), cysteine, cystine and oxidized and reduced glutathione was made. Such a solvent is methyl Cellosolve (2-methoxyethanol; redistilled and peroxide-free) containing 1% (v/v) of 12N-HCl, on either Whatman no. 3MM or no. 1 paper. This solvent, however, does not separate ergothioneine and mercaptohistidine or their corresponding disulphides and we found that propan-2-ol–formic acid (1:1, v/v) was the best solvent system for this separation. R_F values are shown in Table 2. The chromatograms were allowed to run overnight by the ascending–descending technique of Toennies & Kolb (1951), dried at room temperature, suspended for 5 min. in an NH_3 atmosphere in another chromatography tank in order to decompose the disulphide, and blown with a stream of air to remove the ammonia, which gives a background colour, and then developed by dipping in a 0·2% (w/v) ethanolic solution of 2:6-dichloroquinonechloroimide. With ESH and ESSE, brick-red colours develop as soon as the solvent dries from the paper and maximum development occurs after 5 min.; cysteine and reduced glutathione give yellow colours; cystine and oxidized glutathione do not react. Care should be taken not to expose the paper to collidine, lutidine or phenol vapours, and chromatography tanks which have been used previously for these solvents are not satisfactory because they cause the development of a purple background. The atmosphere in some laboratories causes this background to develop slowly but this can be prevented by further developing the chromatogram by dipping in 0·1% (w/v) ninhydrin in anhydrous acetone. The red quinone colours given by ergothioneine are not affected if the ninhydrin colours are allowed to develop overnight at room temperature, but some fading occasionally occurs on heating in the oven. Cysteine and glutathione give the usual ninhydrin reaction superimposed on the yellow quinone colour, and substances such as thiolhistidine and the mixed disulphide of ergothioneine and cysteine

Table 2. *R_F values of various sulphur-containing compounds on Whatman no. 3MM paper by the one-dimensional ascending–descending technique of Toennies & Kolb* (1951)

Substance	R_F in Methyl Cellosolve + 1% (v/v) of 12N-HCl	R_F in Propan-2-ol–formic acid (1:1, v/v)
Ergothioneine	0·44	0·47
Ergothioneine disulphide	0·09	0·50
Cysteine	0·66	0·45
Cystine	0·33	0·17
Mercaptohistidine	0·41	0·22
Mercaptohistidine disulphide	0·09	0·17
Glutathione	0·52	0·48
Cysteinylergothioneine	0·16	—
Glutathionylergothioneine	0·24	—

(ESSC, see below) give both the red quinone and the purple ninhydrin reaction by this procedure. We used the method of Toennies & Kolb (1951) to photograph the chromatograms first after quinone development and again after the final development with ninhydrin.

Preparation of ergothioneine disulphide

Ergothioneine hydrochloride (106·2 mg., 0·4 m-mole) was dissolved in 5N-HCl (4 ml., resulting concentration 0·1M); to this solution was added from a microburette 2N-H_2O_2 (0·2 ml., freshly prepared and standardized), with rapid stirring. Reaction was immediate, as shown by the formation of the yellow colour, paper chromatography and ultraviolet spectra. The solution was evaporated to dryness over NaOH in a vacuum desiccator and the yellow, very deliquescent, residue was dissolved in anhydrous ethanol (dried by distillation from Mg and I_2). The solution was centrifuged to remove traces of any insoluble ergothioneine hydrochloride and the hydrochloride of ergothioneine disulphide was precipitated by the addition of excess of sodium-dried ether, collected by centrifuging and washed with ether; while still moist with ether, the product was placed in a vacuum desiccator and the ether removed in an anhydrous atmosphere. The compound was purified by several reprecipitations from anhydrous ethanol with ether. The pale-yellow, very deliquescent, amorphous

powder, m.p. 198–200° by micro-hot-plate method, was chromatographically pure. Analysis was by Weiler and Strauss, Oxford (Found: C, 41·0; H, 5·7; N, 15·4; S, 12·1; Cl, 13·5. $C_{18}H_{30}O_4N_6S_2Cl_2$ requires C, 40·8; H, 5·7; N, 15·8; S, 12·1; Cl, 13·4%).

Besides being deliquescent, ergothioneine disulphide dihydrochloride is unstable in water; aqueous solutions, used for chromatography, show a progressive formation of ergothioneine, with a corresponding diminution in the ergothioneine disulphide spot. This decomposition can also be seen spectrophotometrically, the spectrum slowly reverting to that of ergothioneine. This reaction is comparatively slow, complete decomposition of a mM-solution of ergothioneine disulphide dihydrochloride taking 3–5 days. For paper chromatography ergothioneine disulphide dihydrochloride should be dissolved in anhydrous formic acid; such solutions are stable for several weeks when kept in the refrigerator.

The slow decomposition of ergothioneine disulphide dihydrochloride in water is not a simple reduction to ergothioneine, as is the reduction with cysteine or glutathione. The decomposition was followed spectrophotometrically by determining immediately, and thereafter at daily intervals, the absorption at 258 mμ of a solution of ergothioneine disulphide dihydrochloride in water, both before and after reduction with cysteine. When decomposition was complete, the absorption at 258 mμ directly measured was between 60 and 70% of the original ergothioneine value and no further increase was brought about by the addition of cysteine. This slow decomposition of the dihydrochloride becomes immediate if the solution is made alkaline. It has consequently not been possible, so far, to prepare the free base.

Ergothioneine disulphide dihydrochloride gives the typical magenta colour for ergothioneine in the Hunter reaction, but this is equivalent to only 65–70% of the expected colour. When pure ergothioneine disulphide dihydrochloride is subjected to column chromatography either on alumina in water–ethanol–formic acid (Melville & Lubschez, 1953) or on ion-exchange resins (Heath & Wildy, 1956), only ergothioneine is eluted, decomposition having taken place as shown by paper chromatography and differential-ultraviolet spectra with and without cysteine.

Ergothioneine disulphide dihydrochloride is stable in ethanol, formic acid or 5N-HCl solution and has been kept for over a year, without decomposition, when stored as a dry powder.

Formation of the mixed disulphides, ergothioneine–cysteine and ergothioneine–glutathione

Ergothioneine hydrochloride (53·1 mg., 0·2 m-mole) and cysteine hydrochloride (31·5 mg., 0·2 m-mole) were dissolved in 5N-HCl (4 ml.); to this solution was added from a microburette 2N-H_2O_2 (0·2 ml., freshly prepared and standardized) with rapid stirring. There was an immediate formation of a yellow colour, as with the preparation of ergothioneine disulphide, which then rapidly disappeared to leave a colourless solution. The reaction mixture was evaporated to dryness in a vacuum desiccator, extracted with anhydrous ethanol, centrifuged, precipitated with sodium-dried ether and purified by repeated precipitation from dry ethanol with dry ether.

Paper chromatography of the product, applied in formic acid solution, revealed only one spot. Neither ESH, cysteine nor ESSE was detectable with dichloroquinonechloroimide and treatment with ninhydrin showed the absence also of cystine. Thus the only product of the reaction is the single spot (R_F 0·16) which gives both the quinone and the ninhydrin reactions.

Treatment of the mixed disulphide ergothioneine–cysteine (ESSC) with cysteine results in the immediate formation of ergothioneine and cystine,

$$\text{ESSC} + \text{cysteine} \rightarrow \text{ESH} + \text{cystine}.$$

The mixed disulphide is even more unstable in water than ergothioneine disulphide itself and chromatograms of fresh aqueous solutions show the presence of ergothioneine and cystine. No cysteine is formed in the decomposition. This progressive liberation of ergothioneine with time can be clearly demonstrated chromatographically. An attempt to isolate the free base, by treatment with sodium acetate, at an earlier stage in the preparation before this decomposition was known, resulted only in the isolation of cystine.

The mixed disulphide of ergothioneine and glutathione was prepared in an analogous manner. Its chromatographic behaviour (R_F 0·24) was similar to that of ESSC when applied in formic acid solution.

The hydrochlorides of ergothioneine–cystine and ergothioneine–glutathione are both colourless, deliquescent, amorphous powders, soluble in ethanol. Neither melts below 300°.

Reaction between ergothioneine disulphide and cystine

Solutions (0·05M in 2N-HCl) of ergothioneine disulphide dihydrochloride and cystine were mixed and kept at 20° for 4 days.

No formation by molecular rearrangement of the mixed disulphide ergothioneine–cysteine could be demonstrated (paper chromatography and ultraviolet spectrum).

DISCUSSION

It has been established that ergothioneine can be oxidized under suitable conditions to the disulphide form, and that this oxidation is reversible: $2\text{ESH} \rightleftharpoons \text{ESSE}$. In the physiological pH range, the equilibrium form is, as far as can be detected, completely in the reduced state. It is only in strongly acid solution, in which the disulphide is more stable, that both forms can exist together.

Evidence for this was discovered during the chromatographic part of the experimental work; we made stock solutions, in formic acid, of ergothioneine and ergothioneine disulphide dihydrochloride for use as markers; whereas the disulphide was stable, and did not decompose to ergothioneine, the solution of ergothioneine was slowly oxidized to the disulphide form, the formic acid solution eventually giving both ergothioneine and ergothioneine disulphide spots on the chromatogram. The reverse is true in aqueous solution; ergothioneine is completely stable but the disulphide decomposes to the free thiol form.

The properties of ergothioneine and thiourea disulphides and mixed disulphides with cysteine are similar but not identical (cf. Toennies, 1937; Pirie, 1933). No evidence for the liberation of free sulphur on decomposition of ergothioneine disulphide was found, whereas dithioformamidine readily decomposes in this manner. Likewise the analogue of the reaction between cystine and dithioformamidine to yield the mixed disulphide *S*-guanylthiocysteine could not be demonstrated between ergothioneine disulphide and cystine, even in the presence of free ergothioneine to catalyse the reaction.

However, the formation of mixed disulphides was possible owing to the fact that the oxidation of cysteine in strongly acid solution by hydrogen peroxide is comparatively slow (Toennies & Gallan, 1939), whereas the oxidation of ergothioneine is extremely rapid; this could be seen by the fleeting formation of the yellow colour due to ergothioneine disulphide formation, just after the addition of the peroxide. Thus when a mixture of cysteine and ergothioneine is oxidized in strongly acid solution by peroxide, the first very rapid reaction is $2ESH + H_2O_2 \rightarrow ESSE + 2H_2O$. But the reaction of cysteine and ergothioneine disulphide ($ESSE + 2$ cysteine $\rightarrow 2ESSC$) is also extremely rapid and this results in the formation of ergothioneine–cysteine mixed disulphide. Evidence in support of this mechanism is given by the chromatograms of the reaction product, which does not contain any of the original compounds, ergothioneine or cysteine, neither does it contain any of their separate oxidation products, cystine or ergothioneine disulphide, all of which are clearly resolved by the solvent and developing reagents. This reaction mechanism also explains the catalysis of the peroxide oxidation of cysteine in acid solution by ergothioneine.

Owing to the instability of ergothioneine disulphide, it is not possible at present to detect its occurrence in biological systems. In blood ergothioneine always occurs in the presence of a large excess of reduced glutathione, which would maintain it in the reduced form. Even if this were not so, the usual methods of isolation from aqueous extracts by either ion-exchange resins or alumina chromatography would themselves bring about the reduction.

The fact that only 60–70% of the theoretical amount of ergothioneine is detected by ultraviolet absorption or Hunter colour reaction after the decomposition of the disulphide in water indicates that the mode of decomposition is not a simple reduction but probably procedes via a hydrolytic fission to the sulphenic acid,

$$ESSE + H_2O \rightarrow ESH + ESOH,$$

which then decomposes further

$$2ESOH \rightarrow ESH + ESO_2H.$$

The sulphinic acids of the mercaptoglyoxalines are very unstable and readily lose sulphur dioxide (Balaban & King, 1937). It must be emphasized that this mechanism is purely hypothetical and no evidence for the existence of the sulphenic acid has been sought.

The reaction of ergothioneine with 2:6-dichloroquinonechloroimide has been found to be by far the most satisfactory means of chromatographic detection. Colour forms only in neutral or faintly alkaline solution, and ergothioneine disulphide dihydrochloride as such gives only a very weak and slow reaction. After neutralization with ammonia the reaction is immediate owing to the decomposition of the disulphide to ergothioneine.

Mercaptohistidine also forms a disulphide which can be separated chromatographically, with methyl Cellosolve–HCl. This compound, like mercaptohistidine itself, gives both the red quinone reaction, after treatment with ammonia, and a ninhydrin reaction. Its properties are analogous to those of ergothioneine disulphide.

At an early stage in this work attempts were made to prepare ergothioneine disulphide by both iodine and permanganate oxidations. Neither method was successful. It is possible that these or other oxidants could be used, but owing to their high ultraviolet absorption it was not possible to follow the oxidation spectrophotometrically, and owing to the lability of ergothioneine disulphide none was isolated.

The fact that it has now been established that ergothioneine can be catalytically oxidized by molecular oxygen or non-catalytically by hydrogen peroxide might be significant in relation to its biochemical function; it might act as a very transitory proton donor and acceptor.

SUMMARY

1. Ergothioneine can be oxidized slowly to the disulphide form by oxygen in the presence of copper and in strongly acid solution but not in neutral or alkaline solution. The rate of oxidation can be followed spectrophotometrically.

2. Ergothioneine is rapidly oxidized to the disulphide form by the equivalent amount of hydrogen peroxide in strongly acid solution in the absence of copper.

3. This oxidation is reversible. Cysteine or reduced glutathione causes immediate reduction.

4. Ergothioneine disulphide dihydrochloride has been isolated, m.p. 198–200°. It is stable when dry or in strongly acid solution. It is unstable in water or in alkaline solution. Its properties have been described.

5. A paper-chromatographic technique is given for the separation and detection of the reduced and oxidized forms of ergothioneine, cysteine and glutathione.

6. Mixed disulphides of ergothioneine with cysteine and glutathione have been prepared.

The greater part of the work described in this paper was carried out in America but it was completed in England, and one of us (H.H.) wishes to thank Mr R. Boardman for technical assistance in the final stages and Burroughs Wellcome Ltd. for a generous gift of ergothioneine.

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The Purity of Thyroglobulin Isolated from Normal and Carcinomatous Thyroid Tissue on one Patient by Fractional Salting-out with Ammonium Sulphate

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Thyroglobulin was prepared (Stanley, 1956) by the method of Derrien, Michel & Roche (1948) from normal and carcinomatous thyroid tissue of a patient (Mrs Ru.) with a follicular carcinoma of the thyroid gland. The patient had received a tracer dose of radio-iodide 2 days before thyroidectomy. Differences in the distribution of ^{131}I between the fractions obtained during the preparation of pure thyroglobulin have been reported and discussed (Stanley, 1956). The homogeneity and question of identity of the thyroglobulins (Ru_N and Ru_A) from normal and carcinomatous thyroid tissue have now been investigated by electrophoresis and by an immunological test.

MATERIAL AND METHODS

The preparation of Ru_N and Ru_A was described previously (Stanley, 1956). Solutions [1% (w/v); 2·5 ml.] of the two proteins in 0·05M-veronal buffer (pH 8·6, I 0·05) were subjected to electrophoresis in the 2 ml. cell of a Perkin–Elmer apparatus, model 38, after dialysis against the buffer at 0–1° for at least 16 hr. This analysis was repeated with 0·4% solutions of both proteins.

The solutions of Ru_N and Ru_A were recovered from the electrophoresis cells and used for immunological analysis by the method of Ouchterlony (1948). Antibodies against Ru_A were prepared in a rabbit.

RESULTS

Figs. 1 and 2 represent the electrophoretic schlieren patterns obtained with 1% (w/v) solutions of Ru_N and Ru_A respectively, at the times indicated on the figures. Both proteins contain a single electrophoretic component, but differ significantly in mobility. The mobilities in the descending (μ_D) and ascending (μ_A) limbs were as follows: Ru_N: μ_D $5{\cdot}38 \times 10^{-5}$, μ_A $5{\cdot}85 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$; Ru_A: μ_D $4{\cdot}97 \times 10^{-5}$, μ_A $5{\cdot}44 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. The difference in mobility was confirmed in a second run with 0·4% solutions of the proteins, when the following mobilities were determined: Ru_N: μ_D $5{\cdot}53 \times 10^{-5}$, μ_A $5{\cdot}71 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$; Ru_A: μ_D $4{\cdot}91 \times 10^{-5}$, μ_A $5{\cdot}09 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$.

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A contact print of the cell in which a gel-diffusion test of anti-Ru_A serum was run against Ru_N and Ru_A is reproduced in Fig. 3. No antigenic component occurs in Ru_A which is not also present, in roughly the same proportion, in Ru_N. This test indicates, moreover, the presence of one major and two, possibly three, minor antigenic components in each protein.

DISCUSSION

Thyroglobulin prepared by the method of Derrien *et al.* (1948) has been shown by the same workers to be microheterogeneous in spite of the fact that it is isolated by fractional salting-out within narrow limits of ammonium sulphate concentration. This microheterogeneity is indicated by breaks in the solubility curve, which suggest the presence of three components. This microheterogeneity has also been found with thyroglobulin prepared by the same method from human thyroid glands (Roche, Michel, Deltour & Michel, 1952). Derrien, Michel, Pedersen & Roche (1949) demonstrated that thyroglobulin from porcine glands is electrophoretically homogeneous.

In the current investigation thyroglobulin from normal and from carcinomatous thyroid tissue of one patient was shown to be immunologically heterogeneous, each protein containing three antigenic components. Moreover the Ouchterlony gel-diffusion test enabled us to conclude that no antigenic components were present in Ru_A which were not also present in Ru_N.

Electrophoretic analysis suggested that Ru_N and Ru_A were homogeneous and had mobilities of the same order as those determined by Derrien *et al.* (1949) for porcine thyroglobulin in phosphate buffer (pH 7·68, I 0·2). The difference in mobility determined for each protein at 1 and 0·4% (w/v) concentration may be due to a difference in viscosity between the more and less concentrated solutions, but the difference between the mobilities

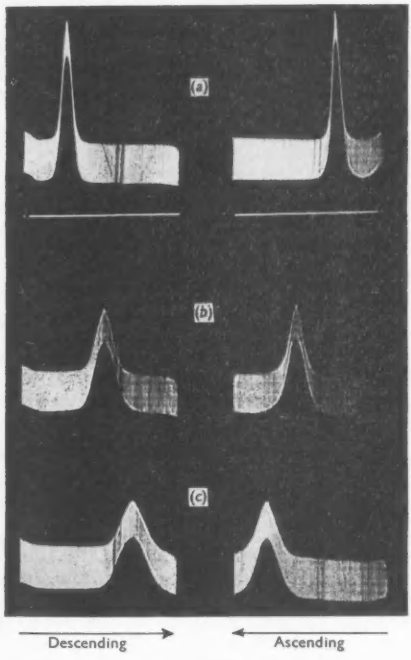

Fig. 1. Electrophoretic schlieren patterns of thyroglobulin (1%, w/v) in 0·05 M-veronal buffer (pH 8·6, I 0·05). This sample of thyroglobulin (Ru_N) was prepared by the method of Derrien *et al.* (1948) from the normal thyroid tissue of a patient with a follicular carcinoma of the thyroid gland. Times at which the photographs were taken are: (*a*) 60 min.; (*b*) 120 min.; (*c*) 165 min.

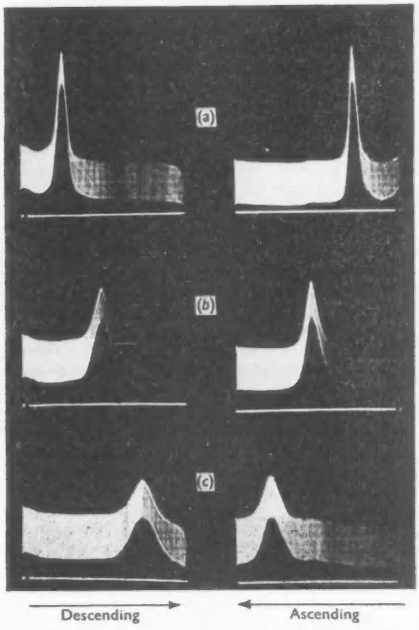

Fig. 2. Electrophoretic schlieren patterns of thyroglobulin (1%, w/v) in 0·05 M-veronal buffer (pH 8·6, I 0·05). This sample of thyroglobulin (Ru_A) was prepared by the method of Derrien *et al.* (1948) from the abnormal thyroid tissue of a patient with a follicular carcinoma of the thyroid gland. Times at which the photographs were taken are: (*a*) 60 min.; (*b*) 120 min.; (*c*) 180 min.

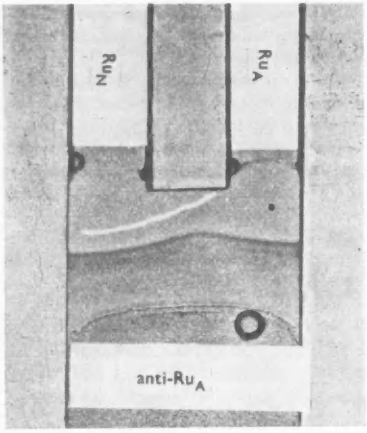

Fig. 3. Gel-diffusion precipitin pattern obtained by the method of Ouchterlony (1948). Solutions (about 1% in 0·05M-veronal buffer, pH 8·6) of thyroglobulin from normal (Ru_N, 0·1 ml.) and abnormal (Ru_A, 0·1 ml.) thyroid tissue of a patient with a follicular carcinoma of the thyroid gland and an anti-serum prepared against Ru_A in a rabbit, were placed in the positions marked Ru_N, Ru_A and anti-Ru_A respectively and allowed to diffuse together. At least three precipitin bands formed; the fourth and lowest band, visible also under Ru_A to the naked eye, was too diffuse to show in the photograph. The white streak is an artifact.

of Ru_N and Ru_A at the same concentration appears to be due to a structural difference which is not reflected in the antigenic properties of the proteins.

SUMMARY

1. Thyroglobulins prepared from normal and carcinomatous thyroid tissue of one patient migrated as single components during electrophoresis in 0·05M-veronal buffer (pH 8·6, I 0·05) at concentrations of 1 and 0·4% (w/v). They differed significantly in mobility.

2. Analysis by the gel-diffusion method of Ouchterlony revealed the presence of three, possibly four, antigenic components in each thyroglobulin but no qualitative difference between the two thyroglobulins.

One of us (P.G.S.) is indebted to the University of Melbourne for a Travelling Scholarship, to the British Council for a Travel Grant and to Professor F. G. Young for the provision of laboratory facilities.

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Biosynthesis of Intestinal Mucins

1. SURVEY OF INCORPORATION OF [^{35}S]SULPHATE BY ISOLATED GASTROINTESTINAL TISSUES

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When [^{35}S]sulphate is injected into mammals most of it is eliminated in the urine and faeces (Dziewiatkowski, 1949). The comparatively small amount of isotope which is retained is incorporated by a number of tissues, notably the epithelium of the gastrointestinal tract and cartilaginous tissue (Lewison, Levi, Jones, Jones & Silberstein, 1951; Dziewiatkowski, 1953; Bélanger, 1954). By the use of radioautographic techniques it has been shown (Bélanger, 1953; Boström, 1953; Davies & Young, 1954; Jennings & Florey, 1956) that in the gastrointestinal tract the various mucin-secreting cells are the principal sites of localization and that here inorganic sulphate is incorporated into sulphated amino polysaccharides (Crevier & Bélanger, 1954; Kent, Whitehouse, Jennings & Florey, 1956).

In order to study in greater detail the biosynthesis of such polymeric substances, which are major organic components of epithelial mucin, a survey has now been made of the utilization of [^{35}S]sulphate *in vitro* by surviving slices and segments of various types of gastrointestinal

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tissue. [The term 'mucin' has the meaning defined by Kent & Whitehouse (1955*a*), throughout this paper.] Previous workers have shown the uptake *in vitro* of this ion by mammalian cartilage (Boström, 1953; Clark & Umbreit, 1954), chick embryo (Layton, 1950*a*; Jones & Gerarde, 1953; Boyd & Neumann, 1954) and granulation tissue (Layton, 1950*b*; Kodicek & Loewi, 1955) and have demonstrated that it becomes incorporated into sulphated polymers.

Part of the present results have already been reported in brief (Pasternak, Whitehouse & Kent, 1955).

METHODS

Preparation of tissues

Sheep and calves. The whole stomach was removed immediately after slaughter, rinsed with cold water and placed in ice-cold saline. The gastric mucosa from the pyloric region of the abomasum (fourth stomach) was separated from the muscular layer, and pieces (20–30 mg. dry wt.) were blotted, weighed and placed in chilled manometer flasks containing ice-cold incubation medium.

Frogs. Frogs were killed by pithing. The stomach was removed and pieces of gastric mucosa prepared as described above. The dry wt. of tissue was 10–20 mg./flask.

Guinea pigs. Adult guinea pigs were killed by cervical fracture. The stomach was removed and pieces of gastric mucosa (45–65 mg. dry wt. per flask) were prepared as described above. Portions of duodenum, ileum and colon were separated from adhering fat and rinsed with ice-cold incubation medium; whole segments (approx. 2 cm. in length) were blotted, weighed, everted and placed in chilled manometer flasks (45–75 mg. dry wt./flask). The liver was used in some experiments, 35–45 mg. dry wt. of thin tissue slices being added to flasks containing gastric or intestinal tissue.

Incubation

The incubation medium used was the 'medium III' described by Krebs (1950) from which the organic acids had been omitted, i.e. a phosphate buffer containing 0·02 M-glucose (total vol. 4 ml./flask). For the experiments with frog gastric mucosa 22 ml. of water was added/100 ml. of this medium. A solution (0·1 ml.) of $Na_2{}^{35}SO_4$ (Radiochemical Centre, Amersham), diluted to contain 1–10 μC, was added. In certain experiments the following additions were made to this medium: sodium acetate, sodium α-oxoglutarate, L-glutamine, (final concn. 0·002 M); a mixture of 20 common amino acids (final concns. 0·004 M for DL-acids and 0·002 M for L-acids and glycine) and aqueous yeast extract A of Whitehouse, Kent, Peters & Foulkes (1954) (0·1 ml. added to 4 ml. of incubation medium). Incubation was at 37° in oxygen for 3 hr. With frog gastric mucosa the temperature was 25°.

Oxygen uptake was measured throughout incubation, carbon dioxide being absorbed by 0·2 ml. of 2 M-sodium hydroxide; values of oxygen uptake (Q_{O_2}) are expressed as μl. of O_2/mg. dry wt. of tissue/hr.

At the end of incubation, the contents of each flask were transferred to centrifuge tubes and heated at 100° for 10 min. Tissue was then separated by centrifuging from the supernatant medium, which contained mucin liberated during incubation.

Extraction of tissues

Tissues after incubation and centrifuging were twice extracted with 10 ml. of 5% (w/v) sodium acetate for 12 hr. at room temp., and the resulting extracts were centrifuged before dialysis.

Dialysis

Incubation medium (4 ml.) and tissue extract (20 ml.) were dialysed in cellophan at 2° against distilled water (2·5 l.) which was changed every 12 hr. Sodium sulphate [0·2 ml. of 5% (w/v)] and a mixture containing 1% of penicillin G and 1% of streptomycin phosphate (0·2 ml.) were added to the dialysing solutions at every change of water. Duplicate samples of the incubation medium (0·05 ml.) and tissue extract (0·2 ml.) were removed before dialysis for radioactive assay; further samples (0·2 ml.) were removed at intervals during dialysis. Dialysis was continued until constant radioactivity of the internal solution was attained (90–130 hr.). At the end of dialysis the volumes of the dialysed medium and tissue extract were measured; for certain experiments both fractions were then freeze-dried. The products isolated after dialysis of the medium and tissue extract are termed 'extruded' and 'extracted' mucin respectively. (The word 'extruded' is not used in any physiological sense, but merely to denote the non-dialysable material liberated from the tissue during incubation.)

Analytical procedures

Dry-weight determination. The dry wt. of tissues was determined after heating duplicate samples for 12 hr. at 105°.

Radioactive assay. Samples (0·05 or 0·2 ml.) of the solutions being dialysed were plated directly on to Perspex disks (1·8 cm.^{2}) and allowed to dry at room temp. before the radioactivity was measured (GM mica end-window tube EHM 2s operating at 1640 v). All counts were corrected for background (approx. 10 counts/min.), self-absorption (by extrapolation to infinite thinness) and dead-time. Allowance for decay of ^{35}S was made by expressing the final radioactivity of extruded and extracted mucin fractions as total counts/min./100 000 counts/min. initially added/mg. of tissue dry wt., abbreviated subsequently to counts/mg. of tissue.

Amino sugar determination. Amino sugar content of freeze-dried material was determined after hydrolysis with 4 N-HCl (4 hr.) or 3 N-HCl (13 hr.) at 100° by the Elson & Morgan (1933) reaction. The modification of Johnston, Ogston & Stanier (1951) was employed, with concentrated reagents as suggested by Exley (1957).

Ester sulphate determination. Ester sulphate was determined by the method of Kent & Whitehouse (1955*b*).

Nitrogen determination. Nitrogen was determined by the micro-Kjeldahl method.

Blood-group specificity. Blood-group specificity was estimated by an iso-agglutination-inhibition technique suggested by Dr R. G. Macfarlane, F.R.S. Two series of doubling dilutions of a 0·5% (w/v) solution of mucin in 0·85% saline were prepared; to one of the series of tubes an equal volume (0·2 ml.) of human anti-A serum was added, and to the other an equal volume of anti-B serum. The contents of each tube were mixed and allowed to stand for ½ hr. at room temp. (20–25°). Human A_1 erythrocytes in

citrate [0·2 ml. of a 5% (v/v) suspension containing 0·5% (w/v) trisodium citrate and 0·85% (w/v) sodium chloride] were then added to the tubes containing anti-A serum, and a similar volume of B erythrocytes to the tubes containing anti-B serum. Both series of tubes were mixed and allowed to stand for 1 hr. at room temperature. At the end of this time the degree of agglutination in each tube was compared with a saline control.

Penetration of tissue by [^{35}S]*sulphate.* The method of Deyrup & Ussing (1955) was used. Tissues were prepared and incubated as described above. The radioactivity of medium and of aqueous tissue extract (5 ml.) was also determined as described above.

RESULTS

Oxygen uptake and yield of mucin. The rate of consumption of oxygen by tissue slices showed a slow regular decline during incubation. Table 1 gives Q_{O_2} values and the corresponding yield of mucin fractions. Owing to the finely divided state of freeze-dried mucins, slight losses were incurred during isolation. The values are therefore to be regarded as minimal figures.

Composition of mucin fractions. Table 2 shows values for the total ester sulphate and amino sugar content of mucin fractions derived after incubation from sheep gastric tissue and guinea-pig colonic tissue. In the latter case 30% of the mucin occurs in the incubation medium, the major part being obtained only by chemical extraction of the tissue. In both the extruded and extracted fractions of guinea-pig colon, ester sulphate and amino sugar are present in the molar ratio of 1:1. This suggests that the mucin fractions contain amino polysaccharides which are sulphated to a considerable degree.

[^{35}S]*Sulphate uptake.* A preliminary investigation with sheep gastric mucosa indicated that this tissue incorporates relatively small amounts of [^{35}S]sulphate under the described conditions of incubation (Table 3). These values were not increased by addition of the following substances to the medium: sodium acetate, L-glutamine, sodium α-oxoglutarate, amino acid mixture or yeast extract. Substitution of the medium by sheep serum was also without effect on uptake of [^{35}S]-sulphate.

Under the same conditions of incubation, calf gastric mucosa showed somewhat greater uptake of [^{35}S]sulphate, particularly in the extruded mucin fraction (1·4 counts/mg. of tissue). Incubation of gastric mucosa of frog and guinea pig, with or without the additional substrates mentioned above, gave no appreciable uptake of [^{35}S]sulphate, even when these tissues were incubated in the presence of liver slices. Control liver slices incorporated [^{35}S]sulphate to a value of 0·7 count/mg. of tissue.

The behaviour of other gastrointestinal tissues was studied. Guinea-pig duodenum, ileum and colon were found to incorporate considerable amounts of [^{35}S]sulphate. Additional substrates or liver slices were without effect on the level of incorporation.

With gastric tissue the mucosa alone was studied, whereas with colon segments of whole intestine were used. Since the musculature appears to be almost inactive in fixing [^{35}S]sulphate (Jennings & Florey, 1956), the results (Table 3) indicate that the mucosa of colon is very much more active than that of stomach in this respect.

Table 1. *Yield of mucin and* Q_{O_2} *of gastrointestinal tissues*

Values are averages derived from several experiments, the numbers being given in parentheses. For experimental details see text. The Q_{O_2} values were calculated from the slope of the Q_{O_2}/time curve at 150 min. The S.E.M. of the results for the sheep gastric mucosae experiments are given.

Tissue	Q_{O_2}	Extruded mucin (μg./mg. of dry tissue)	Extracted mucin (μg./mg. of dry tissue)
Sheep gastric mucosa	−5·5±1·0 (14)	80±40 (10)	220±90 (9)
Calf gastric mucosa	−3·8 (2)	60 (1)	—
Frog gastric mucosa	−1·9 (3)	20 (1)	—
Guinea-pig gastric mucosa	−10·8 (1)	70 (1)	—
Guinea-pig colon	−6·5 (4)	100 (2)	—

Table 2. *Composition of mucin fractions from gastrointestinal tissues*

Values (in μg./mg. of dry tissue)±S.E.M. are averages derived from several experiments, the numbers being given in parentheses. For experimental details see text.

Tissue	Mucin fraction	Ester sulphate	Amino sugar
Sheep gastric mucosa	Extruded	2·4 (1)	3·5±1·3 (9)
	Extracted	1·7 (1)	6·2±2·4 (10)
Guinea-pig colon	Extruded	2·9±1·2 (13)	4·7±0·6 (6)
	Extracted	5·8±1·4 (5)	10·0±0·9 (6)

It was subsequently found that for sheep, as for guinea pig, the uptake of [^{35}S]sulphate by gastrointestinal tissues was in the descending order: colon, duodenum, stomach.

It was decided to examine the behaviour of guinea-pig gastric and colonic tissue in more detail, since the difference in incorporation of ^{35}S by the two tissues is so marked. The ester sulphate content of the freeze-dried extruded fractions was measured and found to be similar (24 μg. of sulphate/mg. of gastric mucin and 22 μg. of sulphate/mg. of colonic mucin); the chemical nature of these mucins in terms of total ester sulphate does not therefore provide an explanation of the differences in fixation of ^{35}S.

Penetration of tissue by [^{35}S]*sulphate.* In view of the low uptake of [^{35}S]sulphate by guinea-pig gastric mucosa compared with colon, the penetration of these tissues by [^{35}S]sulphate was determined. Values, calculated from the relation

$$[SO_4^{2-}]_{\text{tissue water}} = \frac{^{35}S_{\text{tissue water}}}{^{35}S_{\text{medium}}} \times [SO_4^{2-}]_{\text{medium}},$$

where $[SO_4^{2-}]$ is expressed in μmoles/ml. and ^{35}S in counts/min./ml., were found to be similar, namely 0·88 μmole of sulphate in tissue water of gastric mucosa and 0·89 μmole in that of colon segments, compared with 1·40 μmoles of sulphate in the incubation medium. Calculation of the tissue sulphate relative volume,

$$100 \times \frac{\text{(concn. of } ^{35}\text{S in tissue water)}}{\text{(concn. of } ^{35}\text{S in medium)}},$$

gives a value of 64 for guinea-pig colon segments, a figure similar to that (95) found for rat-colon segments by Deyrup & Ussing (1955), in so far as 'active uptake' of [^{35}S]sulphate from the medium is not indicated. Differences of concentration of K^+ ion may account for the higher value found by Deyrup & Ussing (1955).

Blood-group specificity. The results (Table 4) show that gastric mucin fractions are potent inhibitors of isoagglutination, whereas intestinal mucins are inactive.

DISCUSSION

Oxygen consumption. The rates of uptake of oxygen observed with the various tissues studied are in agreement with those reported by previous workers (Davies, 1948; Crane & Davies, 1951; Davenport & Chavré, 1952). Steady respiration by gastric mucosa was observed for longer periods than was noted by Lutwak-Mann (1947). The present results for the Q_{O_2} of guinea pig, pig and sheep tissues are similar to the findings of Dickens & Weil-Malherbe (1941), who studied rat tissues,

Table 3. *Incorporation of* [^{35}S]*sulphate by gastrointestinal tissues*

Values (in counts/100 000 added/mg. of dry tissue) are averages derived from several experiments, the numbers performed being given in parentheses. For experimental details see text.

Tissue	Extruded mucin	Extracted mucin	Total
Sheep gastric mucosa	0·03 (4)	0·02 (4)	0·05
Calf gastric mucosa	1·4 (2)	0·2 (2)	1·6
Frog gastric mucosa	1·0 (3)	0·0 (3)	1·0
Guinea-pig gastric mucosa	0·7 (3)	0·03 (3)	0·73
Guinea-pig duodenum	4·0 (1)	17·5 (1)	21·5
Guinea-pig ileum	8·7 (1)	3·1 (1)	11·8
Guinea-pig colon	37·7 (6)	20·5 (5)	58·2

Table 4. *Inhibition of isoagglutination by gastrointestinal mucins*

The numbers of animals examined are shown in parentheses. For experimental details see text. The sign – means that the sample showed no activity.

Tissue	Group of serum inhibited: Extruded mucin	Group of serum inhibited: Extracted mucin	Highest dilution exhibiting inhibition
Sheep gastric mucosa	A (3)	A (1)	1:2000–1:10 000
Calf gastric mucosa	B (1)	B (1)	1:2000
Frog gastric mucosa	*AB (3)	—	1:30 000
Guinea-pig gastric mucosa	B (2)	B (1)	1:10 000
Guinea-pig duodenum	B (1) (slight)	–(1)	–
Guinea-pig ileum	–(1)	–(1)	–
Guinea-pig colon	–(2)	–(1)	–

* Pooled sample from three frogs.

Table 5. *Composition of gastrointestinal mucins*

All values are mg./100 mg. of mucin.

Tissue	N (%)	Amino sugar (%)	Ester sulphate (%)	Authors
Sheep stomach§	7·7	3·1*	1·7	Present work
Pig stomach	11·7	7·7	0·14†	Werner (1953)
Pig stomach	—	16·7	0·34	Whitehouse (1955)
Pig colon	13·2	5·0	1·0†	Werner (1953)
Guinea-pig colon§	7·3	4·7‡	2·1	Present work

* Confirmed by pyrrole method of Exley (1957).
† Calculated from Werner's (1953) data for fractionated material.
‡ From Table 2.
§ Extruded, freeze-dried mucin.

and found a descending order for the Q_{O_2} values of duodenum, ileum and colon.

Properties of extruded and extracted mucin. Werner (1953) and Whitehouse (1955), in contrast with other authors, have studied gastrointestinal mucins by techniques designed to avoid degradation during isolation; their results are compared with those obtained during the present experiments in Table 5. The low values of amino sugar content observed by Werner (1953) and by us suggest the presence of considerable amounts of non-carbohydrate material, and Werner (1953) was indeed able to increase the amino sugar content of his preparations two- to three-fold by removal of protein by tryptic digestion. Interpretation of the high ester sulphate/amino sugar ratios found in these studies (see also Table 2) must await the results of purification and fractionation of the freeze-dried material.

Blood-group activity (Table 4) was found in the gastric mucins of sheep, calf (cf. Hartmann, 1941; Jorpes & Thaning, 1945; Beiser & Kabat, 1952), frog and guinea pig. Identification of the nature of the blood-group substance from frog is difficult, since the material was obtained after pooling the extruded mucin fractions from three stomachs; it is therefore not possible to say whether the AB activity is due to one frog or whether it represents a mixture of A and B substances from different frogs. Nevertheless, the finding of any A activity is interesting in view of the apparent lack of A specificity in the red blood cells from frogs (Ashhurst, 1956).

Among the mucins of the gastrointestinal tract, only those from stomach appear to exhibit marked blood-group activity (cf. Witebsky & Neter, 1935; Satoh, 1949). Zittle, Smith & Krejci (1948) seem to be the only authors to have isolated intestinal mucins possessing strong blood-group specificity. The possibility that guinea-pig intestinal mucins contain blood-group substances, but in an inactive state, cannot be excluded (cf. Witebsky & Neter, 1935). In view of a recent study relating the presence of blood-group specific substances in the gut of humans to the incidence of duodenal ulcer (Clarke *et al.* 1956), it seems of considerable importance to investigate such compounds further; we have made an attempt to correlate blood-group activity and the chemical composition of mucins produced by different regions of the gastrointestinal tract (Pasternak & Kent, 1957).

Incorporation of [^{35}S]sulphate during incubation. Calculation of incorporation of ^{35}S (in terms of μg. of sulphate) as a fraction of the total ester sulphate content of extruded and extracted mucin (Table 2) gives a value for the proportion of mucin sulphate groups which are newly formed during incubation. In guinea-pig colon this is 4·5% for the extruded fraction and 1·2% for the extracted mucin fraction. Since the sulphate content of other gastric mucins is similar and the uptake of ^{35}S is considerably less, the percentage incorporation in such mucins is even smaller (less than 1%). In all the tissues studied, therefore, most of the extruded or extracted mucin is not synthesized during incubation, but exists preformed at the start of the experiment. It has not been possible to decide whether the newly synthesized material represents a net increase of mucin or whether it is accompanied by an equivalent breakdown (see Pasternak & Kent, 1958).

The mechanism of incorporation of [^{35}S]sulphate will be considered in greater detail elsewhere (Pasternak & Kent, 1958). There does not appear to be a relation between oxygen uptake and sulphate incorporation by different tissues of the gastrointestinal tract (Tables 1 and 3); since sulphate fixation is only one of the many functions exhibited by these tissues during incubation *in vitro*, such a relation is hardly to be expected.

A feature of the present study is the relative inactivity of gastric tissue, as opposed to intestinal tissue, in fixing [^{35}S]sulphate (Table 3). This result is in accord with experiments on incorporation of [^{35}S]sulphate by whole animals: Jennings & Florey (1956), for example, find that several mammalian species exhibit only slight uptake of ^{35}S in the neck cells of gastric epithelium, whereas greater fixation

is apparent in Brunner's glands of duodenum and in goblet cells of the small and large intestine.

The inactivity of gastric tissue is unlikely to be a reflexion of the type of mucin which is produced, since the degree of sulphation of gastric and colonic mucins is similar; it is worth noting, however, that Satoh (1949) has found more 'hydrolysable sulphur' in human colonic mucin than in that from the small intestine.

It is considered improbable, moreover, that lack of uptake of [^{35}S]sulphate by gastric tissue is caused by conditions *in vitro*; permeability of the tissue to [^{35}S]sulphate, for instance, is similar for stomach and colon. Insufficient energy is also unlikely to be the cause, since gastric tissues have a higher Q_{O_2} than intestinal ones (Table 1). An explanation of the low level of incorporation of ^{35}S-sulphate by stomach, in contrast with colon, may lie in differences in the concentrations of certain enzymes or coenzymes within the tissue.

SUMMARY

1. The oxygen uptake and incorporation of [^{35}S]sulphate by isolated tissues of the gastrointestinal tract of several animals have been measured.

2. The incorporation of [^{35}S]sulphate by gastric mucosa of sheep, calf, frog and guinea pig was found to be negligible (less than 2 counts/mg. of dry tissue); uptake by lower regions of sheep and guinea-pig intestine, especially by colon, is considerable (10–60 counts/mg. of dry tissue).

3. The penetration of gastric and colonic tissue by ^{35}S-sulphate has been determined and found to be similar for these two tissues (approx. 60%).

4. The ester sulphate and amino sugar contents of gastric and colonic mucins have been measured.

5. Differences in incorporation of [^{35}S]sulphate by gastric and colonic tissue are not accounted for on the basis of (*a*) penetration of [^{35}S]sulphate into the tissue or (*b*) the degree of sulphation of the mucins produced by the tissue.

6. The blood-group specificity of several types of gastrointestinal mucin has been determined: activity appears to be restricted to mucins isolated from gastric tissue.

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The Effect of Urokinase and Fibrinolytic Euglobulins on Synthetic Amino Acid Esters

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The increasing interest and the accumulating knowledge of fibrinolysis has induced a search for more convenient and accurate methods for the measurement of this particular proteolytic phenomenon. Procedures allowing a more precise measurement of single phases of the complex process would be of great interest.

Fibrinolysis is induced by activating plasminogen, a precursor contained in most human body fluids, to plasmin, the active enzyme. The best known activating agent, until recently almost exclusively used, is streptokinase. Streptokinase acts probably through the intermediary of a proactivator, also contained in most body fluids. It is also known that activating human 'kinases' exist, particularly in urine (Astrup & Sterndorff, 1952). Only recently has it been possible to isolate and purify this human kinase from urine and to make it available in quantities (Kaulla, 1954, 1956). The urinary material is called urokinase or urofibrinolysokinase. It enables the production of fibrinolysis with human activating agents instead of with the bacterial streptokinase.

In order to measure fibrinolytic processes more accurately, the fact has been exploited that proteolytic enzymes are capable of splitting synthetic amino acid esters. Purified plasmin preparations have been shown to split the synthetic amino acid esters (Troll, Sherry & Wachman, 1954; Troll & Sherry, 1955). However, Schultz, Moorman, Matoush & Lincoln (1957) have seen no difference in esterase activity as measured by the release of free amino acids from the esters when blood from patients with very marked spontaneous fibrinolytic activity was compared with non-fibrinolytic normal blood.

This paper presents the results from a study designed to investigate further the correlation of ester-splitting activity with the proteolytic breakdown of fibrin (fibrinolysis). The esterase activity of human urokinase preparations, fibrinolytic human euglobulins and non-fibrinolytic euglobulins, alone and in the presence of plasma, were determined. The fibrinolytic euglobulins were obtained from plasma treated with human urine. When added before clotting they caused, in the concentration used, complete dissolution of a plasma clot in 20 min.–1 hr. The non-fibrinolytic euglobulins were obtained from untreated plasma. They did not cause appreciable lysis of a plasma clot on 24 hr. incubation.

Urokinase acts, not as a fibrinolytic enzyme itself, but as an activating agent towards plasminogen (which in our experiments is contained in the plasma), transforming it into plasmin (fibrinolytic enzyme). On the other hand, the fibrinolytic euglobulins contain active plasmin formed by activation of plasminogen during the treatment of the plasma from which they were derived with urine (see below).

Our results demonstrate a further lack of correlation between esterase activity and fibrinolytic activity when the activation of human-plasma plasminogen was brought about by human activators. This is in contrast with the results obtained after activation of plasma plasminogen by streptokinase. Both findings would allow for speculation whether the intermediate steps in the process of fibrinolysis could be correlated with esterase activity.

MATERIALS AND METHODS

Urokinase. Urokinase was prepared from fresh human urine according to the method described by Kaulla (1956) and stored under vacuum at 4°.

Euglobulins. For the preparation of fibrinolytic euglobulins, 1 vol. of outdated blood-bank plasma was added to 20 vol. of fresh human urine, and the mixture dialysed for 3 hr. against ice-cold running tap water. This treatment brings about the activation of plasminogen contained in the plasma to plasmin by the urine activator. The mixture was then saturated with CO_2. By this step the euglobulins were precipitated and most of the antifibrinolysin was removed. The plasmin was precipitated with the euglobulins (Kaulla & Taylor, 1957).

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The non-fibrinolytic euglobulins were prepared from plasma by diluting it with 20 vol. of distilled water and subsequently saturating with CO_2, which precipitated them.

The precipitates obtained by these procedures were separated by centrifuging and dissolved in buffered saline [0·74 M-sodium bartitol–acetate, pH 7·4, + 0·85% aq. NaCl (1:4, v/v)] to the original plasma volumes. The redissolved euglobulins were stored at −25° and thawed immediately before use.

Plasma. Only human plasma was used throughout the study. It was obtained from freshly drawn, citrated blood [1 vol. of 3·8% (w/v) sodium citrate (U.S.P.) to 4 vol. of blood] by centrifuging at 2000 rev./min. for 10 min.

Streptokinase. Varidase (Lederle Laboratories Division, American Cyanamid Co., New York, N.Y.) was used. The lyophilized preparation was dissolved in distilled water at a concentration of 10 000 units/ml. To each tube 0·5 ml. (5000 units) was added.

Synthetic amino acid esters. Lysine ethyl ester and toluene-*p*-sulphonyl- (tosyl)-arginine methyl ester (Mann Research Laboratories, New York, N.Y.) were each dissolved in distilled water at a concentration of 0·1 M immediately before use.

Estimation of the esterase and fibrinolytic activities of urokinase. The lysine ethyl ester and tosylarginine methyl ester assays were performed in the manner described by Troll & Sherry (1955) with 40 and 60 min. incubation periods. A water blank was run to determine the amount of amino acid liberated by incubation without enzyme. Also, streptokinase, urokinase, a urokinase–streptokinase mixture, and plasma blanks were determined. An amount of urokinase (usually 0·5–2 mg. added before clotting) sufficient to cause lysis of a plasma clot in 20–120 min. at 37° was dissolved in 1 ml. of fresh plasma drawn from a healthy member of the laboratory. A sample of this mixture was diluted 1:4 with distilled water for the amino acid ester assays with and without the addition of streptokinase, and another sample was diluted 1:25 in 0·1 M-$CaCl_2$ solution to bring about clotting in order to determine its effect on a fibrin clot after 45 min. and 24 hr. incubations. The amount of fibrin present after each period of incubation was estimated by its tyrosine content (Quick, 1951).

Determination of esterase and fibrinolytic activities of the euglobulin preparations. 1 ml. of either euglobulin preparation, equivalent to 1 ml. of the original plasma, was diluted 1:4 with distilled water and the esterase activity of the fibrinolytic as well as of the non-fibrinolytic euglobulins was determined with the two ester assays. To determine the effect of the euglobulins in the presence of plasma, 1 ml. of either euglobulin was added to 1 ml. of fresh plasma and the mixture diluted to 5 ml. with distilled water for the ester assays, or to 25 ml. with 0·1 M-$CaCl_2$ for the fibrin method. Both types of euglobulins, either alone or mixed with plasma, were run with and without the addition of streptokinase. At the same time the plasma alone was tested for esterase and fibrinolytic activity with and without addition of streptokinase.

In all esterase determinations, only the results of the 60 min. incubations are presented, since the statistical analysis showed comparable differences of values obtained with the various preparations for the 40 and 60 min. periods.

The significance of differences found with different systems was estimated by the use of Student's *t* test.

RESULTS

Comparison of the esterase and fibrinolytic activities of urokinase. When urokinase was added to plasma and the mixture was clotted, lysis of the fibrin clot occurred in 20–120 min. according to the strength of the urokinase preparation; no increase in esterase activity was observed on the addition of urokinase to plasma in the amino acid ester assays (Table 1). Streptokinase in plasma caused lysis in 1–2 hr., as did the combination of urokinase and streptokinase in plasma. There was no additive effect on fibrinolysis by the combination of these two plasminogen-activating agents. A significant increase in esterase activity was observed on the addition of streptokinase and streptokinase–urokinase combination to plasma in both ester assays. When streptokinase–urokinase–plasma values are compared with the streptokinase–plasma values, the increase in ester-splitting activity is significantly less in the lysine ethyl ester, but not in the tosylarginine methyl ester assay.

By comparing the 45 min. with the 24 hr. incubations for plasma alone in the fibrin method, a mean decrease of 2·7% of fibrin was observed. Plasma alone in the ester assays showed no significant increase in the amount of amino acid liberated over the water blank (Table 1). Also no increase of liberated amino acids was seen with the urokinase, streptokinase, and streptokinase–urokinase blanks.

Table 1. *Effect of urokinase on spontaneous and streptokinase-activated esterase activity in plasma*

Esterase activity is expressed as a mean increase in μmoles of acid liberated during 60 min. incubation. Numbers in parentheses indicate number of determinations. Tosylarginine and lysine figures are expressed as mean ± standard error.

	Tosylarginine	Lysine	Clot lysis time
Water (13)	0·180 ± 0·022	0·018 ± 0·005	—
Streptokinase (13)	0·125 ± 0·029	0·020 ± 0·007	—
Urokinase (4)	0·210 ± 0·075	0	—
Urokinase + streptokinase (4)	0·172 ± 0·021	0	—
Plasma (12)	0·206 ± 0·020	0·022 ± 0·006	> 24 hr.
Plasma + urokinase (12)	0·206 ± 0·046	0·023 ± 0·007	20–120 min.
Plasma + streptokinase (12)	0·580 ± 0·029	0·405 ± 0·040	60–120 min.
Plasma + urokinase + streptokinase (12)	0·540 ± 0·055	0·292 ± 0·030	60–120 min.

Table 2. *Ester-splitting activity of fibrinolytic and non-fibrinolytic euglobulins used alone and with plasma*

Results are expressed as in Table 1.

	Tosylarginine	Lysine	Clot lysis time
Fibrinolytic euglobulin (9)	0·383±0·048	0·018±0·009	—
Non-fibrinolytic euglobulin (9)	0·193±0·031	0·028±0·007	—
Fibrinolytic euglobulin + streptokinase (9)	0·430±0·019	0·112±0·025	—
Non-fibrinolytic euglobulin + streptokinase (9)	0·514±0·046	0·216±0·034	—
Plasma (12)	0·262±0·033	0·029±0·008	>24 hr.
Plasma + fibrinolytic euglobulin (12)	0·246±0·027	0·031±0·009	20–50 min.
Plasma + non-fibrinolytic euglobulin (12)	0·132±0·025	0·036±0·009	>24 hr.
Plasma + streptokinase (12)	0·644±0·047	0·380±0·022	60–120 min.
Plasma + fibrinolytic euglobulin + streptokinase (12)	0·677±0·035	0·418±0·022	20–60 min.
Plasma + non-fibrinolytic euglobulin + streptokinase (12)	0·836±0·043	0·710±0·049	20–60 min.

Enzyme activity of the euglobulin preparations in absence of plasma. The fibrinolytic euglobulins had a marked enzyme activity in the tosylarginine methyl ester assay which is not significantly increased by the addition of streptokinase (Table 2). The fibrinolytic euglobulins showed no activity in the lysine ethyl ester assay, but a significant increase is observed with the addition of streptokinase to them.

The non-fibrinolytic euglobulins exhibited no enzyme activity in either assay. The addition of streptokinase to the non-fibrinolytic euglobulins resulted in an increase of the ester-splitting activity significantly higher than the values obtained with the addition of streptokinase to the fibrinolytic euglobulins in the lysine ethyl ester assay, but not significantly higher in the tosylarginine methyl ester assay.

Effect of the euglobulin preparation in the presence of plasma. When the fibrinolytic euglobulins were added to plasma and the fibrin method was used, they caused a complete lysis of the fibrin clot in 20–50 min. In contrast with this, a mean decrease of only 29·4% of fibrin occurred in 24 hr. with the non-fibrinolytic euglobulins and a decrease of 7·9% with plasma alone. When either of the euglobulins was added together with streptokinase to plasma, complete lysis of the fibrin clot occurred in 20–60 min.

The esterase activity of plasma to which fibrinolytic euglobulins were added was no different from that of plasma alone, though they induced strong fibrinolysis. The non-fibrinolytic euglobulins caused a significant decrease in the tosylarginine methyl ester assay, but there was no change in the lysine ethyl ester assay. With both esters, the increase in activity of fibrinolytic euglobulins in combination with streptokinase was not significantly higher than that with streptokinase alone. The increase in activity of non-fibrinolytic euglobulins together with streptokinase over that with streptokinase alone was highly significant (Table 2).

DISCUSSION

Little correlation could be found between the fibrinolysis-inducing effect of urokinase and fibrinolytic euglobulins and the plasma-esterase activity. The esterase activity and the fibrinolysis phenomenon are possibly two concomitant aspects of plasmin. A previous investigation has shown a lack of correlation between the two (Schultz *et al.* 1957) in the presence of plasma. Lysis of a plasma clot was readily induced by the addition of fibrinolytic euglobulins, urokinase or streptokinase; an increase in enzyme activity, as measured by the splitting of synthetic amino acid esters, was demonstrated here only on the addition of streptokinase.

The lack of increase in esterase activity on the addition of urokinase to plasma possibly might be due to the well-known presence of plasmatic inhibitors (Bastian, Hill & Ercoli, 1956). The decrease in activity on the addition of urokinase together with streptokinase compared with that obtained on the addition of streptokinase alone could be explained either by a competitive effect of the urokinase (which is added to the plasma before streptokinase) on plasminogen to make it no longer available for streptokinase activation, or by the presence of an inhibitory substance for esterase activity of plasmin in the urokinase preparation. A proteolytic inhibitor which will inhibit human plasma enzyme activated with streptokinase has been purified from human urine (Shulman, 1955).

An activation scheme for plasmin has been presented by Müllertz (1955) and Astrup (1956), in which a proactivator is converted by streptokinase into an activator, which in turn activates plasminogen to plasmin. However, no conclusive information is available as to whether this activation scheme holds true for the activation of plasminogen brought about by human 'kinases', or whether the activation induced by them follows another pathway. Troll & Sherry (1955) have concluded that the activator has the ability to split the lysine esters,

whereas plasmin splits both the arginine and lysine esters.

The results presented in this paper indicate that the fibrinolytic euglobulin preparations contain plasmin which splits the tosylarginine ester but not the lysine ester; they contain little plasminogen, since there is no increase in the splitting of the arginine esters with the addition of streptokinase; and they contain some proactivator, as is shown by the increased breakdown of lysine esters after addition of streptokinase. The non-fibrinolytic euglobulin contains little plasmin but considerable amounts of proactivator and plasminogen, which can be converted into activator and plasmin respectively, as is shown by the marked increase in esterase activity in both assays on the addition of streptokinase.

When fibrinolytic euglobulins are added to plasma, the lack of increase of esterase activity could be caused by the inhibitors of the plasma which have been shown to inhibit ester-splitting activity of trypsin (Bastian *et al.* 1956). The significant inhibition of the plasma-esterase activity by the non-fibrinolytic euglobulins could be due to the presence in the euglobulins of inhibitors which might have been eliminated from the fibrinolytic euglobulins during their preparation. Such inhibitors have been demonstrated in precipitated euglobulins (Müllertz, 1953). The significant increase of esterase observed when streptokinase is added to the non-fibrinolytic euglobulins in plasma can be explained by the presence of plasminogen and proactivator in the euglobulin preparation.

SUMMARY

1. Little correlation could be found between the fibrinolysis-inducing effect of urokinase and fibrinolytic euglobulins as shown by lysis of plasma clots and the plasma-enzyme activity measured by the splitting of synthetic amino acid esters.

2. When urokinase was added to plasma there was no increase in esterase activity, but a marked fibrinolytic activity was observed.

3. Fibrinolytic euglobulins used alone showed an activity towards tosylarginine methyl ester, but not towards lysine ethyl ester. There was no increase in activity when the fibrinolytic euglobulins were added to plasma, but a strong fibrinolysis was obtained.

4. It is concluded that the synthetic-substrate methods are not applicable as tests for fibrinolytic activity in the presence of plasma. Possible reasons for the discrepancies are discussed.

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Studies on the Aspartic Decarboxylase of *Nocardia globerula*

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Specific decarboxylases have been described for several amino acids and have proved useful for the estimation of these amino acids in mixtures (Gale, 1957). The enzyme system from *Clostridium welchii* is much more active on glutamic acid than aspartic acid. The determination of aspartic acid with this preparation depends on the difference between the volume of carbon dioxide produced from glutamic acid plus aspartic acid, and that produced from glutamic acid alone when the aspartic decarboxylase is inhibited (Meister, Sober & Tice, 1951). The present communication describes a preparation specific for the decarboxylation of L-aspartic acid and completely inactive on all other amino acids tested.

MATERIALS AND METHODS

Organism. The organism was isolated from soil and corresponded closely in properties with *Nocardia globerula* (Gray, 1928). A strain of this organism has been deposited with the National Collection of Industrial Bacteria, Teddington, Middlesex, under the no. 8852. During these studies the organism was maintained on agar slopes

containing 2% (w/v) of Difco peptone and subcultured once a month.

Preparation of the acetone-dried powder. The inoculum for enzyme preparation was taken from a peptone slope culture grown for 24 hr. at 30° and suspended in 2·5 l. of 2% (w/v) peptone in a 5 l. round-bottom flask. Incubation was continued for 60 hr. with occasional shaking. The culture was then harvested by centrifuging, the pellet washed once with and resuspended in 0·85% NaCl, and pipetted rapidly into 20 vol. of acetone at 4°. The coagulum was removed on a Büchner filter, washed once with acetone (4°) and once with ether (4°). The pad was then broken up and allowed to dry in air at room temperature. The yield of acetone-dried powder was about 0·6 g./l. of culture, with a qCO_2 of 50 ($qCO_2 = \mu$l. of CO_2/mg. of acetone-dried powder/hr.) under the conditions given below. For use the powder was suspended in water (10 mg./ml.). The aspartic decarboxylase activity of the dry powder was stable for at least 2 years in a stoppered bottle at room temperature, and that of the suspension for several months at −15°.

Buffers. The buffers used were made up by titrating 100 ml. of the acid (0·3 M) with the appropriate alkali to the required pH value and diluting to 150 ml. with water. The molarity of the buffer given refers to the concentration of total acid, dissociated and undissociated.

Manometric methods. Determinations of the rate of the reaction were made in single-side-bulb Warburg manometers at 37° with air as the gas phase. The main compartment contained 0·5 ml. of the suspension of acetone-dried powder, sodium acetate buffer (pH 5·5, 0·1 M final concentration) and any other additions required; the side bulb contained 2–10 μmoles of L-aspartic acid in sodium acetate buffer (0·1 M, pH 5·5). The total volume of liquid was 3 ml. The substrate was added after equilibration and readings were taken for at least 20 min. to obtain an estimate of the rate of CO_2 production.

Quantitative estimations of aspartic acid were made in a similar manner except that double-side-bulb manometers were used, the second side bulb containing 0·4 ml. of 2 N-H_2SO_4, which was added after completion of the reaction to expel any bound CO_2. Two controls were set up with the same system but with the aspartic acid-containing solution replaced by water. The first of these contained a paper soaked in KOH soln. in the centre well. The pressure change occurring in this manometer is due mainly to a change in vapour pressure resulting from the addition of the sulphuric acid (Peel, personal communication), and the pressure changes in the other manometers were corrected by subtracting this value. The corrected pressure change in the second control was then converted into a volume and subtracted from the experimental determinations.

RESULTS

Products of the reaction. The gas produced was absorbed by 10% (w/v) KOH and was therefore assumed to be CO_2. The non-volatile product of the reaction was prepared by the action of the enzyme in ammonium acetate buffer (0·1 M, pH 5·5). When CO_2 evolution had ceased acetic acid was added until a flocculent precipitate came down. The precipitate was centrifuged off; the clear solution was evaporated to dryness at 60° in a stream of air and the residue dissolved in 1 ml. of water and again evaporated to dryness. The resulting substance was identified as α-alanine by its R_F values, in comparison with an authentic sample of α-alanine, on paper chromatograms developed in the ascending direction on Whatman no. 4 paper with the butan-2-ol–formic acid–water and the phenol–aq. NH_3 soln.–water systems of McQuillen & Roberts (1954) and the ethyl methyl ketone–propionic acid–water system of Clayton & Strong (1954). α-Alanine and β-alanine have different R_F values in the last two solvent systems. They also give characteristically different colours when sprayed with a solution of ninhydrin (0·1%, w/v) in acetone containing $CoCl_2$ (0·3%, w/v) and heated to 100° for a few minutes (modified from Wiggins & Williams, 1952), α-alanine giving a purple colour and β-alanine a reddish-brown colour. The residual aspartic acid in the product was separated on paper chromatograms in the phenol–aq. NH_3 soln.–water system (McQuillen & Roberts, 1954) and estimated by the quantitative ninhydrin reaction (Cocking & Yemm, 1954). Less than 0·1% of the initial aspartic acid remained.

The optical rotation of the product was determined in a solution of 140 mg. in 10 ml. of acetic acid in a 2 dm. tube. The rotation was $[\alpha]_D^{25°} + 30°$ (± 2°) and was similar to that of an authentic sample of L-α-alanine.

Quantitative nature of the reaction. The mean of ten determinations of the CO_2 evolved from 10 μmoles of L-aspartic acid was 9·8 μmoles (S.D. 0·6 μmole). The quantitative ninhydrin reaction (Cocking & Yemm, 1954) of the mixture remained unchanged during the reaction, so that α-alanine was apparently produced quantitatively from aspartic acid. Production of CO_2 from protein hydrolysates was also studied and, as shown by Table 1, the volume of CO_2 produced from hydrolysates of pure edestin was close to that calculated on the basis of its aspartic acid content. The volume of CO_2 produced from DL-aspartic acid was half that produced from an equimolar amount of L-aspartic acid (Fig. 1).

Specificity. The preparation produced CO_2 from L-aspartic acid but not from L-alanine, L-arginine, L-cysteine, L-glutamic acid, glycine, L-histidine, DL-isoleucine, L-leucine, L-lysine, DL-methionine, L-ornithine, DL-phenylalanine, L-proline, L-tyrosine, DL-serine, DL-tryptophan, DL-threonine, L-valine, α-L-glutamyl-L-aspartic acid and γ-L-glutamyl-L-aspartic acid. The addition of each of the above amino acids had no effect on the production of CO_2 from L-aspartic acid. It was concluded that the enzyme was specific for L-aspartic acid. The overall reaction is therefore: L-aspartic acid → L-α-alanine + CO_2.

Table 1. *Estimation of aspartic acid in protein hydrolysates*

Pure edestin [254 mg., aspartic acid content 12% (w/w), Tristram, 1953] was hydrolysed with 6N-HCl in a sealed tube for 18 hr. at 105°. The HCl was removed in a stream of air at 45° and the residue dried *in vacuo* three times. The volume was then made up to 10 ml. with water and samples (0·5 ml.) were taken for aspartic acid determination with the *Nocardia globerula* aspartic decarboxylase. Each manometer contained 0·5 ml. of the acetone-dried-powder suspension and sodium acetate buffer (pH 5·5, 0·1M final concentration) in the main compartment; one side bulb contained the protein hydrolysate and the other 0·4 ml. of 2N-H_2SO_4. The hydrolysate was added after equilibration and the H_2SO_4 after completion of the reaction. The estimation was carried out at 37° with air as the gas phase. After allowing for ash and moisture content the expected volume of CO_2 from the aspartic acid in the protein was 227 μl. The value given for the observed volume of CO_2 is corrected for the two controls as described in the text. Control 1 (+KOH) = 6 mm.; control 2 = 17 μl.

	(*a*) Theoretical CO_2 (μl.)	(*b*) Observed CO_2 (μl.)	$(b \times 100)/a$	Mean
1	227	213	94	
2	227	218	96	
3	227	224	98	95
4	227	214	94	
5	227	206	91	

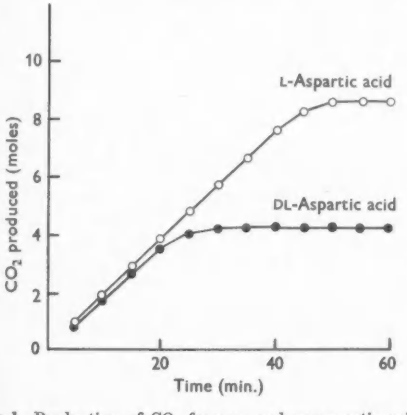

Fig. 1. Production of CO_2 from L- and DL-aspartic acid. Each manometer contained 0·5 ml. of acetone-dried-powder suspension in sodium acetate buffer (pH 5·5, 0·1M final concentration) in the main compartment; the side bulb contained 10 μmoles of L-aspartic acid or 10 μmoles of DL-aspartic acid in the same buffer. Total volume of liquid was 3 ml.; gas phase, air; temperature, 37°. The substrate was added at time 0 after equilibration and readings were taken every 5 min. for 60 min. The results have been corrected for the CO_2 produced in the control without substrate.

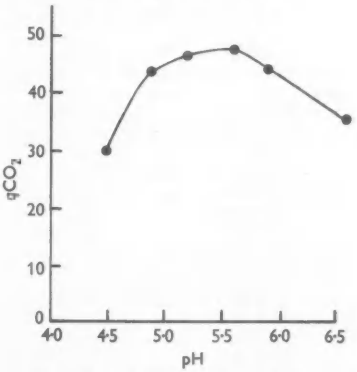

Fig. 2. Effect of pH value on the rate of CO_2 production. Conditions were as described in Fig. 1 except that sodium acetate buffers of the stated pH values were used (0·1M final concentration) and the side bulb contained 10 μ-moles of L-aspartic acid in all cases. The results have been corrected for the control without substrate ($qCO_2 = 3·0$, pH value 5·5) and the rates were calculated over the first 20 min.

Effect of pH. The optimum pH value for CO_2 production from L-aspartic acid was found to be 5·5 under the conditions given above (Fig. 2).

Effect of inhibitors. The effect of various buffers and other additions on the rate of CO_2 production is shown in Table 2. In general the results are similar to those obtained with the *Cl. welchii* system (Meister *et al.* 1951).

Coenzyme requirements. Many of the amino acid decarboxylases have been shown to require pyridoxal phosphate as coenzyme. The aspartic decarboxylase of *Nocardia globerula* was stimulated about 40% by the addition of pyridoxal phosphate (3 μg./ml.). However, it was not possible to resolve the preparation into apoenzyme and coenzyme by treatment with ammoniacal ammonium sulphate, in contrast with certain of the other amino acid decarboxylases (Gale, 1946). Ultraviolet irradiation increased the dependence on added pyridoxal phosphate although the enzyme activity was rapidly lost during this treatment. It seems that pyridoxal phosphate may be the coenzyme for this reaction.

Mechanism of the reaction. The effects of semicarbazide and keto acids are consistent with a reaction mechanism involving transamination, possibly:

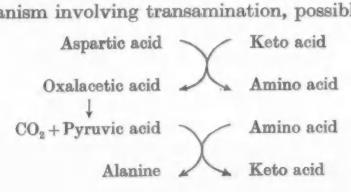

Table 2. *Effect of buffers and other additions on the rate of production of carbon dioxide from* L-*aspartic acid*

Each manometer contained 0·5 ml. of acetone-dried-powder suspension in sodium buffer of the acid stated (pH 5·5, 0·1 M final concentration) in the main compartment; the side bulb contained 10 μmoles of L-aspartic acid in the same buffer. Total volume of liquid was 3 ml. Rates were determined at 37° with air as the gas phase. The substrate was added at time 0 after equilibration. Rates are given after subtraction of the control without substrate, relative to acetate buffer = 100. qCO_2 with acetate buffer = 52.

Buffer	Other additions	Relative rate
Phosphate	—	51
Succinate	—	8
Phthalate	—	5
Barbiturate	—	2
Citrate	—	2
Acetate	Citrate (0·01 M)	2
Acetate	Citrate (0·01 M) + $CaCl_2$ (0·01 M)	2
Acetate	Citrate (0·01 M) + $MgCl_2$ (0·01 M)	3
Acetate	Ethylenediaminetetra-acetate (0·01 M)	100
Acetate	Semicarbazide (0·006 M)	11
Acetate	Semicarbazide (0·006 M) + pyruvate (0·018 M)	140
Acetate	Semicarbazide (0·006 M) + α-ketoglutarate (0·018 M)	138
Acetate	Cetyltrimethylammonium bromide (0·006 M)	1

Table 3. *Decarboxylation of* [^{15}N]*aspartic acid*

Six samples of 10 μmoles of [^{15}N]aspartic acid were decarboxylated by the *Nocardia globerula* aspartic decarboxylase in ammonium acetate buffer (pH 5·5, 0·1 M) at 37° in manometers with air as the gas phase. To three of the samples (*a*) 100 μmoles of L-glutamic acid were added; other samples (*b*) had no addition. The alanine produced was isolated, the amino nitrogen converted into gaseous nitrogen and the ^{14}N:^{15}N ratio determined with a mass spectrograph.

		Ratio ^{14}N:^{15}N	Mean	Atoms % excess
Initial aspartic acid		42·7	—	0·78
Alanine (*a*)	1	44·0	43·9	0·75
	2	46·7		
	3	41·1		
Alanine (*b*)	1	47·1	46·2	0·70
	2	43·1		
	3	48·4		

If an excess of a suitable keto acid were added to the reaction system it might be expected to give rise to the corresponding amino acid. This was tested by adding an excess of α-ketoglutaric acid and estimating the glutamic acid present by the specific decarboxylase method (Gale, 1946). No increase could be detected during the reaction.

A further study was made with [^{15}N]aspartic acid. If the reaction takes place by a transamination mechanism it might be possible to dilute the ^{15}N appearing in the alanine by carrying out the reaction in the presence of another amino acid. Accordingly, six samples of 10 μmoles of [^{15}N]-aspartic acid were decarboxylated by the acetone-dried powder in ammonium acetate buffer (pH 5·5, 0·1 M) at 37° in manometers. To three of the samples 100 μmoles of L-glutamic acid was added. When CO_2 evolution had ceased each sample was treated as follows. The contents of the manometer cup were washed out the protein was removed and the reaction product dried as described for the isolation of α-alanine. The alanine was then separated from the reaction mixture on paper chromatograms in the phenol–aq. NH_3 soln.–water system (McQuillen & Roberts, 1954); the alanine spots, located by the position of marker spots, were cut out and eluted with water. The samples were then dried and made alkaline with methanolic sodium borate (Connell, Dixon & Hanes, 1955) and dried again to remove ammonia. The amino nitrogen was converted into ammonia by digestion with H_2SO_4 and a mercury catalyst (Hiller, Plazin & Van Slyke, 1948). The ammonia formed was distilled off in a Rittenberg all-glass still and collected in 0·1 N-H_2SO_4. After reduction of the volume to 2 ml., ammonium sulphate was added to bring the total ammonium nitrogen to 1 mg./sample. The ammonia was converted into gaseous nitrogen by the method of Sprinson & Rittenberg (1949) with sodium hypobromite. ^{14}N:^{15}N ratios were determined in the mass spectrometer. The initial [^{15}N]-aspartic acid was digested directly in the same way. Table 3 shows that there was little effect of decarboxylation or of the presence of glutamic acid on the ^{14}N:^{15}N ratio of the reaction product. Had the amino group of the glutamic acid equilibrated completely with that of the alanine formed, the product would have contained about 0·07–0·08 atoms % excess of ^{15}N, as the amount of glutamic acid present was ten times the amount of aspartic acid present, and the ^{14}N:^{15}N ratio of the product would have been affected proportionately. It therefore remains uncertain whether the reaction takes place by a direct decarboxylation or by a

transamination mechanism. However, if a transamination reaction is taking place it is apparently concerned with some amino acid other than glutamic acid.

SUMMARY

1. Acetone-dried powders of a strain of *Nocardia globerula* possess aspartic decarboxylase activity but are inactive towards all other amino acids tested.

2. The enzyme can be used for the quantitative estimation of L-aspartic acid in protein hydrolysates and is stable for long periods.

I am indebted to the Medical Research Council for a Scholarship for Training in Research Methods. My thanks are also due to Dr S. V. Perry for a gift of [^{15}N]aspartic acid, to Mr B. E. Boon for the operation of the mass spectrometer and to Mr M. W. Rees for a gift of edestin.

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Succinate as a Respiratory Inhibitor in *Tenebrio molitor* Pupal-Tissue Preparations

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When changes in the chemical composition of *Tenebrio* tissues during the pupal period were being observed (Patterson, 1956*a*) an effort was made to relate these findings to alterations in enzyme pattern which have been reported by various authors, e.g. Agrell (1953) for holometabolous insects during metamorphosis. Furthermore, as citrate was present in greatest concentration in *Tenebrio* pupal tissues at the stage of development corresponding to the lowest rate of oxygen consumption by the live pupa (Patterson, 1956*b*), any variations in the activities of the enzymes of the tricarboxylic acid cycle during metamorphosis were considered to be of interest.

The oxidation of succinate by *Tenebrio* tissues is the only step in the tricarboxylic acid cycle so far investigated. It has been found, surprisingly, that the addition of succinate to pupal-tissue homogenates does not uniformly stimulate respiration and, indeed, that with tissues obtained late in the pupal period a distinct inhibition is produced by adding succinate.

The investigation reported here shows that this diminished respiration was probably due to the inhibition by the succinate of enzymes of the phenolase type, which become more active in pupal tissues towards ecdysis (Dennell, 1944; Karlson & Wecker, 1955; Wojtczak, 1956), and may make a quantitatively important contribution to the endogenous respiration by acting as a general end-system for oxidative processes (cf. Wojtczak, 1955). The oxidation of tyrosine by homogenates of *Tenebrio*-pupal tissues has been shown to be inhibited by succinate and, in the absence of phenolase activity (suppressed by the addition of ethylenediaminetetra-acetic acid), it was demonstrated that succinate was oxidized throughout metamorphosis although not at a uniform rate.

The inhibition of tyrosine oxidation by succinate was studied in acetone-dried-powder preparations, in which the endogenous respiration and succinoxidase activity were negligibly small but the tyrosine activity was high.

MATERIALS AND METHODS

Insects. Larval, pupal and adult tissues of *Tenebrio molitor* were used in these experiments. Fresh pupae were collected from a culture of larval insects (mealworms) and the metamorphosis was allowed to proceed in separate glass jars at 24° until the insects were required. The 'period

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of metamorphosis' as defined by Patterson (1957) lasted 10 days.

Homogenates. Insect tissues were minced with scissors and then homogenized with five times the weight of ice-cold water in an all-glass homogenizer (Potter & Elvehjem, 1936). Fragments of cuticle were carefully excluded by straining through fine gauze. An average 1:5 homogenate contained (in 1 ml.) 45 mg. of total solids and approx. 4·2 mg. of total nitrogen.

Acetone-dried-powder preparations of late-pupal tissue. Tenebrio pupae (89 g.; age not less than 5 days) were macerated with ice-cold water (270 ml.) in a previously cooled Waring Blendor. The resulting pulp (320 ml.) was strained through fine gauze and 10 vol. of ice-cold acetone were stirred in. The precipitate was filtered off at the pump after 30 min. The filter cake was thoroughly washed first with ice-cold acetone and then with cold ether. After air-drying the powder was finally desiccated *in vacuo* over liquid paraffin. An off-white powder (15·9 g.) was obtained. All operations were carried out in a room kept at 5°. For enzyme experiments 1 g. of acetone-dried powder was stirred with 10 ml. of water in an all-glass homogenizer.

Measurements of oxygen uptake. These were made in a Warburg microrespirometer. A 1:5 homogenate was diluted, 1 ml. to 3 ml., with sodium phosphate buffer (pH 7·4; final concn. 0·05M). Uptake of oxygen was measured over a period of not less than 30 min. with air as the gas phase in all experiments.

The acetone-dried powder (0·5 ml. of a 10% suspension in water), diluted with phosphate buffer in the same way, showed only a small respiration (4–7 μl. of O_2/hr.). This could be neglected when the powder was being used as a source of phenolase.

All experiments described in this paper were carried out at 25° in phosphate buffer at pH 7·4.

RESULTS

During the course of some preliminary experiments, pupal-tissue homogenates were prepared in an isotonic sucrose (0·2M) homogenizing medium. For late-pupal tissue an average Q_{O_2} of about 2·67 was obtained for endogenous respiration at 25°. (This compares favourably with the values given in Table 1 for homogenates prepared with water.)

Table 1. *Endogenous respiration of pupal-tissue homogenates at* 25°

Homogenate (1 ml.) in phosphate buffer at pH 7·4. Final concn. of phosphate, 0·05M. Final vol., 3 ml. Centre well of Warburg vessel contained 0·1 ml. of 20% KOH.

Age of pupal tissue (days)	Initial linear rate of O_2 uptake (μl. of O_2/hr.)	Initial Q_{O_2} (μl. of O_2/mg. dry wt./hr.)
0–1	118	2·6
	128	2·8
3–5	38	0·8
	50	1·1
	40	0·9
8–10	121	2·7
	158	3·5
	172	3·8
10 (during ecdysis)	192	4·3
	252	5·6

A simple separation of cell constituents into nuclei plus cellular debris, mitochondria and a supernatant fraction was carried out by the differential-centrifuging technique used by Rees (1954) for insect-muscle tissue. Of the separate tissue fractions prepared, only the final supernatant fraction exhibited a significant endogenous respiration. This amounted to at least one-half of the total respiration of the original homogenate.

The respiration of an homogenate of susceptible pupal tissue was depressed by added succinate and at the same time melanin-pigment formation was suppressed. Therefore it seemed likely that succinate, when it exerted an inhibitory effect, did so by acting upon tyrosinase or a similar enzyme. Being a water-soluble enzyme (Dawson & Tarpley, 1951) tyrosinase would be found in the supernatant fraction separated from pupal tissues.

Aqueous tissue extracts buffered at pH 7·4 were subsequently used in the present work in order to study the respiration of the water-soluble fractions and the inhibition of tyrosinase activity by succinate.

Respiration of pupal-tissue homogenates

When measured in 0·05M-phosphate buffer, pH 7·4, at 25°, the rate of endogenous oxygen uptake shown by the tissue homogenate was approximately constant for no longer than 30 min. and usually for only about 15 min. The substitution of a more complete medium such as that described by Rees (1954) in his metabolic studies of locust muscle did not prolong the period of linear oxygen uptake.

With the simple phosphate-buffer medium, the initial linear rate of oxygen utilization was dependent upon the age of the pupal tissue being used. This is shown in Table 1.

The increased respiration of pupal tissue at the beginning and at the end of metamorphosis is in accord with the familiar U-shaped curve of change in the rate of oxygen utilization by live metamorphosing holometabolous insects (Needham, 1929; Patterson, 1956*b*).

Effect of adding succinate to Tenebrio*-tissue homogenates*

As shown in Table 2, the addition of succinate to tissue homogenates gave rise to variable results, the magnitude and nature of the effect depending upon the stage of metamorphosis at which the tissue was used. The ratio of oxygen consumption in the presence of added succinate (measured in the first 30 min.) to that in the absence of added substrate was calculated from experimental data

obtained with homogenates of tissues of different stages of development and is referred to here as the 'stimulation coefficient'.

It can be seen that the actual depression of respiration by added succinate was apparently limited to homogenates of late-pupal tissues, and that homogenates of tissues obtained at other stages of development apparently oxidized succinate normally. At all stages of development the respiration of tissue homogenates in the presence of succinate was characterized by a linear oxygen uptake lasting, in some cases, for 2 hr.

At the end of some of these experiments, especially where late-pupal tissue had been used, the appearance of the contents of the two 'endogenous flasks' differed quite markedly from that of the flasks to which succinate had been added. Normally, freshly prepared homogenates of *Tenebrio* tissues were off-white to light brown in colour. After 30 or more minutes in a Warburg respirometer at 25°, there was usually considerable formation of a dark melanin-like pigment. Where differences were observed between 'endogenous' and 'succinate' flasks, the contents of the latter flasks were the less pigmented.

With graded amounts of added succinate, 1 ml. samples of 1:5 pupal-tissue homogenates showed maximum inhibition of respiration at a final concentration of about 0·1 M-succinate, whereas at about the same concentration phenolase activity (indicated by melanin-like pigment formation) was minimal (see Table 3).

Table 2. *Effect of added succinate on the respiration of* Tenebrio*-tissue homogenates*

Succinate (100 μmoles) was added to the medium which contained 1 ml. of homogenate. Conditions were as described under Methods.

Stage of development of tissue	Stimulation coeff.*
Larva	1·86
	1·88
	2·41
Pupa 3–5 days	1·49
	1·26
	1·36
Pupa 8–10 days	0·66
	0·96
	0·80
Pupa (during ecdysis)	0·46
	0·50
Adult	1·44
	1·58

* Ratio of rate of O_2 uptake with succinate to rate without succinate.

Table 3. *Inhibition of respiration and of phenolase activity in* 1 *ml. of late-pupal-tissue homogenate by added succinate*

Final concn. of added succinate (M)	Initial rate of respiration (μl. of O_2/hr.)	Melanin-like pigment formed
Nil	310	+ + + + +
0·033	130	+ + + +
0·066	60	+
0·100	38	—

Table 4. *Effect of adding succinate on the respiration of homogenate in the presence of ethylenediaminetetra-acetic acid*

Succinate (100 μmoles) was added to medium containing 1 ml. of homogenate and 50 mM-EDTA. Conditions were as described under Methods.

Stage of development of tissue	Stimulation coeff.*
Larva	1·38
Pupa: 0–1 day	1·16
Pupa: 3–5 days	1·07
Pupa: (during ecdysis)	1·31
Adult	1·19

* See footnote to Table 2.

Effects of ethylenediaminetetra-acetic acid in the absence and in the presence of succinate

The addition of succinate apparently caused an inhibition of pupal-tissue phenolase activity, and it seemed likely that the inhibition of the endogenous respiration by succinate (which reaches a maximum of 88%) might be due to its action on phenolase. Therefore, since phenolase is a copper-containing enzyme (Wigglesworth, 1949; Dawson & Tarpley, 1951) it was anticipated that ethylenediaminetetra-acetic acid (EDTA), which strongly binds copper (see Schwarzenbach, Gut & Andregg, 1954), would bring about a similar diminution of endogenous respiration by the elimination of phenolase activity.

This was found experimentally to be the case. An endogenous respiratory rate of 165 μl. of O_2/hr. was reduced to 32 μl./hr. by 10 mM-EDTA, and to 28 μl./hr. by 30 and by 50 mM-EDTA, giving a maximum inhibition of 82%. At the same time 'melanin' formation was abolished.

In view of the fact that succinate inhibited the endogenous respiration of pupal-tissue homogenates during the latter stages of metamorphosis, it was not possible to assess directly the succinoxidase activity of tissue samples. With maximum inhibition of the phenolases by EDTA, however, the respiration of *Tenebrio*-tissue homogenates was found to be stimulated by the addition of succinate at all stages of tissue development (see Table 4). The degree of stimulation was taken as an index of the succinoxidase activity of the tissue samples.

In no case was the stimulation of respiration as great as that obtained in the absence of EDTA at stages of development where stimulation would occur (see Table 2). Clearly, at this concentration EDTA was partly, at least, inhibiting enzyme systems other than the phenolases, and it would have been better to have used a more specific inhibitor of tyrosinase such as α-naphthylthiourea (DuBois & Erway, 1946).

Inhibition of pupal-tissue tyrosinase by succinate

Tyrosine, when added to *Tenebrio* pupal-tissue homogenates, was readily oxidized, but when succinate was also present the tyrosine was oxidized at a reduced rate (Table 5). The minimum rate of oxidation was obtained with a concentration of 0·12–0·15 M-succinate.

With aqueous suspensions of acetone-dried powders of pupal tissues, the initial rate of tyrosine oxidation was found to decrease logarithmically with concentrations of up to about 0·13 M-succinate when maximum inhibition was obtained (Table 6). This is in close agreement with the result for the inhibition of tyrosinase in homogenates and with the earlier finding that maximum inhibition of the endogenous respiration of late pupal-tissue homogenates was obtained with about 0·1 M-succinate.

The inhibition of tyrosinase by succinate is not peculiar to this one acid. Inhibition of the oxidation of tyrosine by pupal-tissue acetone-dried powder is caused also by malonate, citrate and α-oxoglutarate (Table 7).

The succinate inhibition was selected for somewhat closer study. With a constant level of 100 μmoles of succinate/flask, the initial rate of tyrosine oxidation catalysed by 50 mg. of pupal-tissue acetone-dried powder was obtained graphically from manometric data for substrate concentrations of 0·66–3·96 mM. For comparison, similar data were obtained for uninhibited tyrosine oxidation. Two experiments were carried out with different suspensions prepared from the same stock of acetone-dried powder.

Following the theory of enzyme action of Michaelis & Menten (1913), the relationship between v, initial reaction velocity, S, substrate concentration, and the constants V, the maximum initial-reaction velocity at infinite substrate concentration, and K_m, the Michaelis constant, may be expressed in the form:

$$S/v = S/V + K_m/V.$$

Accordingly, the values of the ratio of substrate concentration to the corresponding initial velocity of the tyrosinase reaction (S/v) were plotted against the substrate concentration (S) for the two sets of conditions and, where applicable, average values of duplicate determinations were used. Two straight lines were calculated as regression lines of S/v upon S (Fig. 1). The slopes for the uninhibited and succinate-inhibited oxidation of tyrosine are $+1·19 \pm 0·18$ and $+1·81 \pm 0·16$ (95% limits) respectively, and are numerically equal to 10^3 times the reciprocals of the respective maximum initial velocities $1/V$ and $1/V'$. The ratios of the Michaelis constants to the maximum initial velocities are given by the intercepts on the ordinate. Thus $K_m/V = 1·80$ and $K'_m/V' = 2·27$, whence the values of K_m and K'_m are $1·59 \times 10^{-3}$ and $1·25 \times 10^{-3}$ respectively. The calculated maximum initial velocity was reduced from 839 μl. of O_2/hr. (V) to 553 μl. of O_2/hr. (V') in the presence of 33 mM-succinate, the difference being significant.

Table 5. *Inhibition by succinate of tyrosinase activity in* 1 *ml. of late-pupal-tissue homogenate*

Tyrosine (8 μmoles) was added. Conditions were as described under Methods.

Concn. of succinate (M)	Initial rate of oxidation (μl. of O_2/hr.)
Nil	221
0·08	117
0·12	86
0·15	82

Table 6. *Inhibition by succinate of tyrosinase activity in pupal-tissue acetone-dried powder*

Tyrosine (10 μmoles) was added. Conditions were as described under Methods.

Concn. of succinate (mM)	Initial rate of oxidation (μl. of O_2/hr.)
Nil	490
0·33	493
5·0	444
9·9	416
17·0	367
33·0	300
61·0	241
133·0	197

Table 7. *Inhibition of tyrosinase activity in pupal-tissue acetone-dried powder by some di- and tricarboxylic acids*

Tyrosine (10 μmoles) was added. Conditions were as described under Methods.

Inhibitor	Concn. (M)	Initial rate of oxidation (μl. of O_2/hr.)
Nil	—	525
Succinate	0·033	433
Malonate	0·033	348
Citrate	0·033	266
α-Oxoglutarate	0·007	69

It is clear that this is not a case of competitive inhibition because, where competition between substrate and inhibitor for the same enzyme site exists, the theoretical maximum reaction velocity at infinite substrate concentration will be unaltered by the presence of an inhibitor. Here, the inhibitor is not a structural analogue of the substrate and competition for the same enzyme sites would not be expected.

From theoretical considerations of the general case of enzyme inhibition by an inhibitor which may combine with both the free enzyme and the enzyme–substrate complex, the following rate equation may be deduced (cf. Bray & White, 1957):

$$V/v' = K_m/S(1 + I/K_I) + (1 + I/K_{SI}),$$

where V is the calculated maximum initial velocity of the reaction in the absence of the inhibitor, v' is the initial inhibited-reaction velocity and S and I are the substrate and inhibitor concentrations. K_m is the Michaelis constant for the enzyme–substrate complex and K_I and K_{SI} are the dissociation constants of the enzyme–inhibitor complex and the enzyme–substrate–inhibitor complex respectively.

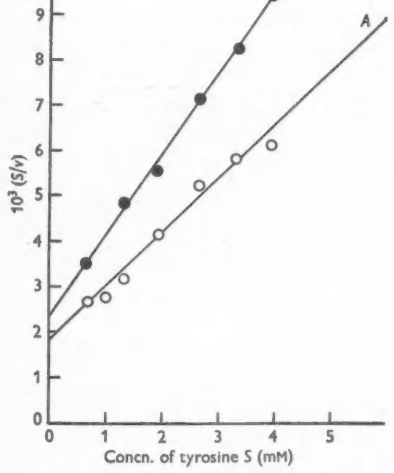

Fig. 1. Inhibition of the tyrosinase activity of 50 mg. of pupal-tissue acetone-dried powder, in 0·05 M-phosphate at pH 7·4 and 25°, caused by adding 100 μmoles of succinate. Total vol., 3 ml. ○, Average experimental points obtained during the uninhibited oxidation of tyrosine (curve *A*); ●, average experimental points for the inhibited oxidation (curve *B*).

Values for V/v' were plotted against the corresponding values for K_m/S, the figures for V and K_m, calculated from curve *A*, Fig. 1, being used. A straight line was drawn through the experimental points, which had a slope of $1{\cdot}24 \pm 0{\cdot}12$. The intercept at the ordinate had a value of $1{\cdot}50 \pm 0{\cdot}12$ (95% limits). The 95% confidence limits for the numerical values obtained for the slope and the intercept did not overlap and were therefore significantly different. This excluded non-competitive inhibition where $K_I = K_{SI}$. Both values were greater than unity and consequently I/K_I and I/K_{SI} were greater than zero. Competitive and uncompetitive types of inhibition were thus ruled out, since in these cases the values of K_I and K_{SI} respectively are very large and the expressions $(1 + I/K_I)$ and $(1 + I/K_{SI})$ are approximately equal to unity (Bray & White, 1957). The inhibition of pupal tyrosinase by succinate was therefore considered to be in all probability one of the general cases of enzyme inhibition.

The dissociation constant of the enzyme–inhibitor complex (K_I), calculated from the observed slope of the graph in Fig. 2, is approx. 139×10^{-3}, and K_{SI}, the dissociation constant of the enzyme–substrate–inhibitor complex, was calculated from the value of the intercept to be approx. $66{\cdot}6 \times 10^{-3}$. Thus succinate when acting as an inhibitor has a greater affinity (about twice) for the tyrosinase–substrate complex than for the free tyrosinase.

Values for V and K_m obtained from experimental observation of uninhibited tyrosine oxidation

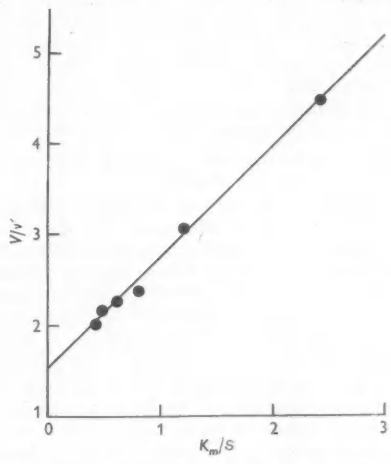

Fig. 2. Plot of V/v' against K_m/S for the oxidation of 0·66–3·96 mM-tyrosine by pupal-tissue acetone-dried powder in the presence of 33·3 mM-succinate.

Table 8. *Initial rates of tyrosine oxidation by pupal-tissue acetone-dried powder inhibited by succinate*

Succinate (100 μmoles) was added. Conditions were as described under Methods.

Concn. of tyrosine (mM)	Mean initial rate of oxidation (observed) (μl. of O_2/hr.)	Mean initial rate of oxidation (calculated) (μl. of O_2/hr.)
0·66	187	187
1·32	274	291
1·98	356	338
2·64	370	418
3·30	400	400
3·96	422	420
	Mean difference	= 7·5
	S.D. of mean difference	= 22·7

(curve *A*, Fig. 1) and the values for K_I and K_{SI} obtained graphically from Fig. 2 were finally substituted in the general equation for enzyme inhibition. The initial velocities of inhibited tyrosine oxidation were calculated for tyrosine concentrations of from 0·66 to 3·96 mM in the presence of 33·3 mM-succinate. They were in good agreement (see Table 8) with the mean experimental values obtained with acetone-dried powders of pupal tissue (i.e. 102·2 % of the observed figures; mean difference 7·5, S.D. 22·7). In confirmation of the results obtained by the graphical methods, the experimental data for the inhibition of pupal tyrosinase by succinate appear to conform to the general equation for enzyme inhibition.

DISCUSSION

Succinate acts as a respiratory inhibitor for late-pupal tissue (i.e. pupal tissue of age more than 5 days at 24°). It is apparently at this stage that the titre of phenolase is highest if this insect resembles some other holometabolous insects cited in the literature (Dennell, 1944; Karlson & Wecker, 1955; Wojtczak, 1956).

If, as has been suggested by Heller (1947) and by Wojtczak (1955), the phenolases behave as general end-systems for oxidations in insects and these phenolase-coupled oxidations account for a large proportion of the endogenous respiration, then the demonstrable antagonism between succinate and tyrosine will account for the large diminution (about 88 %) in the endogenous respiration when succinate is added to late-pupal-tissue homogenates. The addition of EDTA to homogenates brings about a similar degree (82 %) of maximum inhibition. Although the mechanism of action of EDTA is almost certainly different, it is the phenolase-coupled respiration which is largely eliminated, as shown by the inhibition of melanin-pigment formation. In late-pupal tissue of *Tenebrio* therefore it is estimated that respiration coupled to tyrosinase or other phenolases or to them both amounts to about 85 % of the total.

This is not to say that succinate is not utilized at all. In fact, all *T. molitor* tissues were found to catalyse the oxidation of succinate in the presence of sufficient EDTA to abolish phenolase activity.

At other stages of development it is probable that the titre of the phenolases is lower than in the later stages of pupal life. Thus although some of the added succinate would inhibit tyrosinase activity, a larger proportion would also be vigorously oxidized by succinoxidase present (Ludwig & Barsa, 1956), which would account for the net increase in oxygen uptakes observed.

The fact that a number of organic acids besides succinic act as inhibitors of the enzyme tyrosinase is of interest because the extracellular fluids of insects contain, by mammalian standards, a high concentration of organic acids [e.g. in the blood of *Gastrophilus intestinalis* larva there are 58 m-equiv. of organic acids/l. (Levenbook, 1950)]. At such concentrations *Tenebrio*-pupal tyrosinase inhibition was found to be almost maximum. It will therefore be interesting to determine whether the content of organic acids in insect extracellular fluid is decreased when tissue-phenolase activity increases. In this way it will be possible to decide whether the inhibitory action of organic acids such as succinate on tyrosinase is likely to be of significance *in vivo*.

SUMMARY

1. Succinate was oxidized by *Tenebrio molitor* tissue preparations at most stages of development, but late in the pupal period the endogenous respiration of tissue homogenates was greatly reduced by added succinate.

2. The inhibition was probably due to the abolition of phenolase activity, which is at a high titre at the later stages of pupal life.

3. With suspensions of acetone-dried powder as a source of pupal enzyme, the inhibition of tyrosinase by succinate was readily demonstrated. The inhibition followed a general rate equation for enzyme inhibition.

4. Malonate, citrate and α-oxoglutarate also inhibit pupal tyrosinase.

5. Since about 85 % of the endogenous respiration of late-pupal tissue may be abolished either by succinate or ethylenediaminetetra-acetic acid, both of which inhibit tyrosinase activity, it is suggested that this proportion of the endogenous respiration is due to oxidations coupled to a phenolase end-system.

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Studies on Human Synovial Membrane *in vitro*

THE METABOLISM OF NORMAL AND RHEUMATOID SYNOVIA AND THE EFFECT OF HYDROCORTISONE

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Previous metabolic studies *in vitro* have shown that normal synovial membrane is a tissue with an extremely low oxidative metabolism, though its aerobic and anaerobic glycolysis are readily measurable. The presence of rheumatoid disease in the synovial membrane is accompanied by a marked increase in metabolism. The highest rate of carbohydrate metabolism is found in tissues where the villous proliferation which occurs in this disease is most intense. As the proliferative process wanes and the general reaction becomes more fibrotic during remission, the metabolic activity of the synovium diminishes (Dingle & Thomas, 1956). To investigate this increase in oxidation and glycolysis further, the metabolic effects of various intermediates of the glycolytic pathway and the citric acid cycle have been studied.

Articular biopsies of rheumatoid synovia have been performed before and during the administration of cortisone (Hench, Kendall, Slocumb & Polley, 1950). These authors reported that in each case synovial inflammation appeared to be reduced by this hormone, as indicated by a reduction in the cellular reaction, with a decreased number of lymphocytes and plasma cells, and a reduction in papillary villous tufting. Since the metabolic effects of cortisone and hydrocortisone in man are qualitatively similar (Laidlaw, Dingman, Arons, Finkenstaedt & Thorn, 1955) and hydrocortisone is the more potent when administered locally (Hollander, 1951; Boland, 1955), the effect of the latter corticosteroid on synovial metabolism has been investigated. Preliminary observations have shown that hydrocortisone inhibits oxidation and glycolysis of rhematoid synovial villi *in vitro* (Thomas & Dingle, 1955). This work has now been extended to include the effect of this hormone on synovial tissues of varying metabolic activity, and in the presence of various substrates.

METHODS

Synovial tissue. 'Normal' synovial tissue was obtained at the time of operation from patients undergoing menisectomy. These cases had a traumatic aetiology and the effusion had subsided before operation. There was usually some residual subsynovial engorgement of the capillaries on histological examination.

Rheumatoid synovial tissue was similarly obtained from patients undergoing synovectomy or arthrodesis of the knee. All rheumatoid synovia used in this study were shown to have the histological changes characteristic of the disease.

Immediately after excision the synovial tissue was placed in ice-cold Krebs–Ringer-phosphate (Umbreit, Burris & Stauffer, 1951). The chilled tissue was dissected to remove the underlying fat and fibrous tissue from the membrane, and to separate the villi if present. Free villous processes of

0·25–0·5 mm. diam., or membrane strips 5 mm. × 2 mm. × 0·2 mm., after washing for 2–3 min. in ice-cold Krebs–Ringer-phosphate, were placed in Warburg flasks. This washing removed the majority of soluble nitrogenous material. Incubation for 1 hr. at 37° resulted in a very small increase of nitrogen in the suspending fluid, e.g. the average loss of nitrogen to the filtrate from samples containing approx. 3 mg. of nitrogen was less than 0·01 mg.

In some experiments synovial tissues were depleted of endogenous substrates by pre-incubation in an oxygenated Krebs–Ringer-phosphate medium for 1 hr. at 37° after the method of Terner (1952). After the addition of the tissue and immediately before incubation, 0·5 ml. of the suspending fluid was removed for zero-time estimations on the various substrates, or metabolites, in each flask.

Splenic tissue. This was obtained from young male rats immediately after killing. It was kept ice-cold until use.

Tissue suspensions. Villous synovial tissue was cut into small fragments and homogenized in 0·145 M-KCl and 0·024 M-$KHCO_3$ with a motor-driven all-glass homogenizer. The suspension was filtered through glass wool to remove fibrous-tissue fragments. No intact cells were seen on microscopical examination. This preparation was diluted to give a final tissue concentration of about 10% (w/v). Splenic suspensions were similarly made and all preparations were used immediately.

Incubation media. In all aerobic and most anaerobic experiments on whole tissue, Krebs–Ringer-phosphate was used as the basal medium. In experiments where glycolysis was followed manometrically Krebs–Ringer-bicarbonate (Umbreit *et al.* 1951) was employed. Unless otherwise stated, substrates were used at a final concentration of 10 mM. Details of media used for tissue suspensions are given in the text.

Substrates. Commercial preparations of glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1:6-diphosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.), pyruvic acid, α-oxoglutaric acid, oxaloacetic acid, fumaric acid, succinic acid, malic acid, acetic acid and *cis*-aconitic acid (L. Light and Co.) were used as neutral solutions. Diphosphopyridine nucleotide and hexokinase were supplied by Sigma Chemical Co.

Incubation technique. For aerobic experiments oxygen was used as the gas phase, and for anaerobic experiments either nitrogen or nitrogen + carbon dioxide (95:5). The gas was introduced either by a rapid evacuation procedure, or by gassing the chilled flasks for 10 min. All flasks had a 10 min. equilibration period at 37° before manometric measurements were begun. The incubation period was 1 hr. unless otherwise specified. Conventional Warburg flasks were used, with a final fluid volume of 3 ml. For aerobic experiments 10% KOH was included in the central well. The agitation rate was 120 strokes/min. Approx. 30 mg. dry wt. of tissue, which corresponded to 3 mg. of tissue nitrogen, was used per flask.

Chemical analyses. After incubation the tissue was separated from the suspending fluid by filtration through glass wool. The tissue was washed and dissolved in 30% KOH. Samples were taken for estimation of total tissue nitrogen by the micro-Kjeldahl method (Hawk, Oser & Summerson, 1952) and glycogen by the method of Walaas & Walaas (1950). Little glycogen was leached into the suspending medium during incubation, e.g. approx. 0·2–0·4 μg. of glucose equivalent/mg. of tissue nitrogen was found as precipitable material in the filtrate after incubation. The filtrate volume was made up to 9 ml. and protein precipitated by the addition of 1·5 ml. of 5% (w/v) $ZnSO_4,6H_2O$ and 1·5 ml. of approx. 0·3 N-$Ba(OH)_2$. The latter was standardized so that 4·7–4·8 ml., when added to 5 ml. of the $ZnSO_4$ solution, was just pink to phenolphthalein. Glucose was estimated by the method of Nelson (1944), lactic acid by the method of Barker & Summerson (1941), glucose 1-phosphate and glucose 6-phosphate as reducing sugar by the method of Umbreit *et al.* (1951), fructose 6-phosphate and fructose 1:6-diphosphate by a modification of the method of Roe (1934). The results of all estimations were expressed on a tissue-nitrogen basis, the rate of oxygen uptake ($q_{O_2}^{O_2}$) being μl. of O_2 uptake/hr./mg. of tissue nitrogen.

Hormone addition. Hydrocortisone (17-hydroxycorticosterone) was used. The preparation was a suspension in a saline medium (Merck). The suspending medium at the same dilution was present in the control flasks. Recent experience with various methods of rendering corticosteroids soluble (e.g. dissolving in ethanol, propane-1:2-diol or autoclaving or by using the hemisuccinate or dimethyl glutarate esters) has shown that such preparations may give rise to widely different results. In this work we have used one method only, by autoclaving at 20 lb./sq. in. for 10 min. (Miller, 1954).

RESULTS

These results were obtained from the synovia of ten normal subjects and 50 patients with rheumatoid arthritis.

Glycogen. We have been unable to measure accurately the glycogen content of normal synovial tissue owing to its low concentration and the small amount of tissue available at one time. In rheumatoid villous synovia, however, the change in endogenous tissue glycogen during incubation depended upon its initial glycogen content. These results are given in Table 1. When the initial glycogen level was high, and in the absence of substrate, considerable glycogenolysis occurred. When the initial glycogen content was low, however, and in the presence of 10 mM-glucose, a certain amount of glycogen synthesis took place. With a high initial glycogen, under similar conditions, no change in glycogen content was found. One rheumatoid villous tissue, when incubated alone or in the presence of 10 mM-glucose, gave markedly dissimilar rates of glycogenolysis. The effect of hydrocortisone on the changes in tissue glycogen is also given in Table 1. The presence of the hormone resulted in a marked increase in glycogenolysis, or a diminution of glycogen synthesis where this occurred.

Glucose. Under aerobic conditions, hydrocortisone was found to inhibit markedly glucose utilization (Thomas & Dingle, 1955). This inhibition has also been found to occur under anaerobic conditions. For example, in a tissue whose aerobic glucose utilization was 280 μg./mg. of tissue

nitrogen/hr., the addition of 250 μg. of hydrocortisone/ml. resulted in a utilization of 227 μg./mg. of tissue nitrogen/hr. Under anaerobic conditions the same tissue utilized 530 μg./mg. of tissue nitrogen/hr., and in the presence of hormone this was lowered to 346 μg./mg. of tissue nitrogen/hr.

Hexose phosphates. Villous rheumatoid synovia readily metabolized glucose 1-phosphate, glucose 6-phosphate and fructose 1:6-diphosphate, under aerobic conditions. In a typical experiment 800 μg. of glucose 1-phosphate, 300 μg. of glucose 6-phosphate and 450 μg. of fructose 1:6-diphosphate, were utilized/mg. of tissue nitrogen/hr. The endogenous $q_{O_2}^{O_2}$ did not appear to be affected by the addition of glucose 6-phosphate, fructose 6-phosphate or fructose 1:6-diphosphate, and the inhibition of $q_{O_2}^{O_2}$ by hydrocortisone in the presence of these substrates was of a similar degree to that found with glucose as substrate. In one experiment glucose 1-phosphate appeared to increase the rate of oxygen uptake, but the $q_{O_2}^{O_2}$ was again inhibited in the presence of hydrocortisone. The effect of hydrocortisone on the catabolism of the hexose phosphates, under our experimental conditions, has so far yielded erratic results. It seemed possible that the action of hydrocortisone on the metabolism of endogenous substrates might mask the effect of the hormone on the substrates added experimentally. In an attempt to investigate this possibility further, we have examined the effect of hydrocortisone on a rheumatoid synovium, part of which was depleted of endogenous substrates by the method of Terner (1952). These results are given in Table 2. Depleted tissues utilized more glucose but produced less lactate than did non-depleted tissues. Hydrocortisone inhibited glucose utilization and lactate production in both the depleted and non-depleted tissue. With pyruvate as substrate, more lactate was produced by the non-depleted tissue, and under these conditions there was again inhibition by hydrocortisone. In the depleted tissue, however, hydrocortisone showed no inhibitory effect on lactate production. The $q_{O_2}^{O_2}$ in the presence of pyruvate was not appreciably affected by tissue depletion; with glucose as substrate, however, it was lowered. The inhibition of $q_{O_2}^{O_2}$ by hydrocortisone was little affected by these experimental conditions.

Table 1. *Glycogen changes of rheumatoid synovia during incubation and the effect of hydrocortisone*

Hydrocortisone was used at a final concentration of 250 μg./ml. +, Indicates synthesis of glycogen by the tissue; −, indicates glycogenolysis. The number of determinations made on each tissue is given in brackets. Gas phase, O_2. Medium, Krebs–Ringer-phosphate. Temperature, 37°.

Initial tissue glycogen (μg. of glucose equiv./mg. of tissue N)	Substrate (glucose) concn. (mM)	Change in tissue-glycogen content (μg./mg. of tissue N/hr.)	Change in tissue-glycogen content in the presence of hydrocortisone (μg./mg. of tissue N/hr.)
67	0	−27 (2)	−43 (2)
49	0	−22 (8)	−25 (4)
49	5	−11 (3)	−26 (4)
27	5	−13 (6)	—
67	10	−7 (2)	−29 (2)
60	10	0 (3)	—
23	10	0 (3)	—
16	10	+9 (3)	−6 (3)
3	10	+13 (3)	+6 (3)

Table 2. *Effect of depletion upon rheumatoid synovial metabolism*

Figures in brackets are the numbers of determinations. Gas phase, O_2. Medium, Krebs–Ringer-phosphate. Temperature, 37°. Depleted tissue was incubated for 1 hr. at 37°; stored tissue was kept for 1 hr. at 0°.

Experimental conditions					
Treatment	Substrate (10 mM)	Concn. of hydrocortisone (μg./ml.)	$q_{O_2}^{O_2}$	Increase in lactic acid (μg./mg. of tissue N/hr.)	Utilization of glucose (μg./mg. of tissue N/hr.)
Depleted	Sodium pyruvate	0	23 (4)	8·0 (2)	—
Depleted	Sodium pyruvate	250	14 (2)	9·0 (2)	—
Stored	Sodium pyruvate	0	24 (4)	25 (5)	—
Stored	Sodium pyruvate	250	15 (2)	18 (5)	—
Depleted	Glucose	0	15 (3)	135 (3)	560 (3)
Depleted	Glucose	250	12 (3)	100 (3)	310 (3)
Stored	Glucose	0	21 (3)	150 (3)	330 (3)
Stored	Glucose	250	16 (3)	100 (3)	240 (3)

It has been shown in Table 1 that the initial glycogen content of rheumatoid synovium varies widely. This would appear to limit the usefulness of the depletion technique, as pre-incubation for a standard period would not necessarily cause the final carbohydrate levels of different tissues to be similar.

Production of lactate

The aerobic production of lactate by rheumatoid synovia (chemical estimation), with glucose as substrate, has been shown previously to be inhibited by hydrocortisone. The aerobic lactate formed in the presence of the various hexose phosphates is apparently similarly inhibited by hydrocortisone. In view of evidence from the depletion experiments, with pyruvate as substrate, these findings are of dubious value, since it is not known how much lactate is formed from endogenous sources, the metabolism of which could show inhibition by hydrocortisone.

Under anaerobic conditions the increased lactic acid production of rheumatoid synovia is again inhibited by hydrocortisone; for example, the production of 112 μg. of lactate/hr./mg. of tissue nitrogen in the control flasks and 95 μg. of lactate/hr./mg. of tissue nitrogen in the flasks containing 250 μg. of hydrocortisone.

These findings have been confirmed by measuring the rate of acid production in Krebs–Ringer-bicarbonate solution. The rates of anaerobic acid production and the effect of the addition of hydrocortisone are shown in Fig. 1. The rate of acid production by the control flasks was linear for at least 100 min. This finding was similar to that for the rate of oxygen uptake of the tissue, which was also linear for at least 2 hr., provided that glucose was present as a substrate.

In view of the hormonal inhibition of anaerobic lactate production by whole-tissue preparations it was felt that further information as to the nature of this inhibition might best be obtained by the use of broken-cell preparations. Initial experiments were made with synovial-tissue suspensions in bicarbonate–saline. Under these conditions the rate of anaerobic acid production fell off very rapidly, and it was impossible to decide whether hydrocortisone exerted an inhibitory effect. In an endeavour to produce better experimental conditions the addition of various substrates and co-factors were made to the medium. It is evident from Table 3 that the presence of diphosphopyridine nucleotide (DPN) was essential for a rapid rate of glycolysis. Though nicotinamide had only a slight effect on glycolysis by itself, its presence

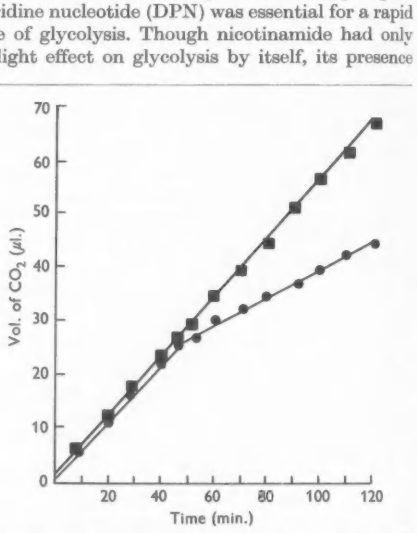

Fig. 1. Effect of hydrocortisone on the anaerobic acid production of synovial tissue. Hydrocortisone (250 μg./ml.) was added from the side arm after incubation for 45 min. Medium, Krebs–Ringer-bicarbonate; glucose (10 mM). Temperature, 37°. Gas phase, $N_2 + CO_2$ (95:5). ■, Control; ●, hydrocortisone added after 45 min.

Table 3. *Anaerobic glycolysis of rheumatoid synovial homogenates*

The basic medium contained KCl (0·154 M), $KHCO_3$ (0·024 M) and $MgCl_2$ (0·01 M). Additions: glucose (0·01 M), nicotinamide (0·05 M unless otherwise specified), DPN (2×10^{-4} M), pyruvate (0·01 M), hexokinase (20 units). 1 ml. of 10% homogenate in bicarbonate–saline. Temperature, 37°. Gas phase, $N_2 + CO_2$ (95:5).

Flask no.	Additions	Time (min.) ...	$q^{N_2}_{CO_2}$ (μl./mg. of tissue N/hr.) 0–15	15–30	30–45	45–60
1	Glucose		0	0	0	0
2	Glucose, nicotinamide (0·01 M)		0·9	0·9	0·9	0·9
3	Glucose, nicotinamide		4·1	2·0	1·6	1·2
4	Glucose, DPN, nicotinamide		20·5	15·6	5·9	2·9
5	Pyruvate		8·9	6·7	3·3	2·2
6	Pyruvate, nicotinamide		9·2	3·1	2·6	2·0
7	Pyruvate, DPN, nicotinamide		7·6	4·7	3·8	3·8
8	Pyruvate, glucose, DPN, nicotinamide		13·0	5·2	3·5	3·5
9	Glucose, hexokinase, nicotinamide		11·2	2·8	1·9	1·4
10	Glucose, pyruvate, hexokinase, DPN, nicotinamide		15·8	6·3	3·2	3·2
11	Glucose, DPN		17·9	5·8	2·1	0·5

with DPN tended to stabilize the rate of glycolysis. In the presence of glucose, neither pyruvate nor hexokinase appeared to be essential. The presence of DPN and nicotinamide had no influence on the rate of acid production with pyruvate as substrate. Further results are presented in Table 4. A bicaronate–saline containing glucose and DPN was used as the basic medium for these experiments. No stabilization of glycolysis occurred when the concentration of nicotinamide was increased. Fructose 1:6-diphosphate alone appeared to inhibit the rate of glycolysis, but in the presence of phosphate and nicotinamide this compound gave a high rate of glycolysis with somewhat improved stabilization. Further experiments with this medium (Table 5, medium no. 4) have in several cases given linear rates up to 45 min. Although this medium has given the best results to date it probably does not represent the optimum conditions for the glycolysis of synovial homogenates. We have tested the effect of hydrocortisone on glycolysis in the presence of this improved medium. The results are given in Table 5. Under these conditions no inhibitory action of hydrocortisone could be demonstrated. The action of hydrocortisone on other tissue homogenates is also given in Table 5. It is evident that the acid production by a rat-spleen suspension in bicarbonate–saline (medium no. 1) is markedly inhibited by hydrocortisone. In the presence of nicotinamide no significant depression of acid production occurred in the first 15 min. With a fortified medium (no. 3) the rate of acid production was inhibited to a lesser degree. Significantly different values were obtained only after incubation for 30 min. Suspensions of rat kidney in the same medium gave similar results.

Oxidative metabolism

The effect on the $q_{O_2}^{O_2}$ of normal and rheumatoid synovia of malate, fumarate, succinate, acetate, pyruvate and glucose is given in Table 6. Although the addition of acetate, pyruvate or glucose had no effect on the $q_{O_2}^{O_2}$ of normal membrane, the addition

Table 4. *Anaerobic glycolysis of rheumatoid synovial homogenates*

The basic medium contained KCl (0·154 M), $KHCO_3$ (0·024 M), $MgCl_2$ (0·01 M), glucose (0·01 M), DPN (2×10^{-4} M). Additions: fructose 1:6-diphosphate (0·03 M), potassium phosphate, pH 7·4 (3 mM), nicotinamide. 1 ml. of 10% (w/v) homogenate in bicarbonate–saline. Temperature, 37°. Gas phase, $N_2 + CO_2$ (95:5).

Flask no.	Additions	Time (min.) ...	$q_{CO_2}^{N_2}$ (μl./mg. of tissue N/hr.) 0–15	15–30	30–45	45–60
1	Nicotinamide (0·05 M)		31·0	18·2	12·7	12·7
2	Nicotinamide (0·01 M)		35·6	25·0	16·0	10·7
3	Fructose 1:6-diphosphate, nicotinamide (0·03 M)		14·2	11·8	9·5	9·5
4	Potassium phosphate		48·0	13·9	4·0	4·0
5	Potassium phosphate, nicotinamide (0·01 M)		21·7	15·5	10·9	9·3
6	Potassium phosphate, fructose 1:6-diphosphate, nicotinamide (0·03 M)		31·2	26·8	19·3	16·4

Table 5. *Effect of hydrocortisone on anaerobic glycolysis of various tissue homogenates*

The basic medium contained KCl (0·154 M), $KHCO_3$ (0·024 M), $MgCl_2$ (0·1 M), glucose (0·01 M). Medium no. 1: basic medium. Medium no. 2, basic medium with nicotinamide (0·02 M). Medium no. 3, basic medium with nicotinamide (0·02 M), potassium phosphate (0·003 M), hexokinase (20 units), adenosine triphosphate (0·001 M), DPN (2×10^{-4} M), pyruvate (0·01 M). Medium no. 4, basic medium with potassium phosphate, fructose 1:6-diphosphate (0·03 M), nicotinamide (0·03 M), DPN (2×10^{-4} M). 1 ml. of 10% (w/v) homogenate. Temperature, 37°. Gas phase $N_2 + CO_2$ (95:5). Figures in parentheses are standard error of mean. Values for % P are given where the probability of the difference between hormone and control results being due to chance is 5% or less.

Tissue	Medium no.	Hormone addition (μg.)	$q_{CO_2}^{N_2}$ (μl./mg. of tissue N/hr.) 0–15 min.	% P	15–30 min.	% P	30–45 min.	% P	45–60 min.	% P
Rat spleen	1	0	24·0 (±2·8)	5·0	18·8 (±2·7)	5·0	15·7 (±1·3)	1·0	13·6 (±1·6)	1·0
		500	14·9 (±1·3)		10·6 (±0·9)		9·1 (±0·7)		7·2 (±0·5)	
Rat spleen	2	0	12·2 (±1·2)		7·8 (±0·8)	1·0	—		—	
		500	9·6 (±0·7)		4·0 (±0·4)		—		—	
Rat spleen	3	0	14·7 (±0·7)		12·0 (±0·6)		11·0 (±0·4)	0·1	10·5 (±0·2)	0·1
		500	14·2 (±0·6)		9·2 (±0·8)		8·5 (±0·2)		8·2 (±0·2)	
Rat kidney	3	0	17·2 (±0·4)	5·0	8·6 (±0·4)	5·0	4·9 (±0·4)	5·0	4·0 (±0·2)	1·0
		500	16·1 (±0·8)		5·8 (±0·7)		2·9 (±0·3)		1·3 (±0·2)	
Rheumatoid synovium	4	0	44·6 (±1·4)		39·6 (±3·3)		35·4 (±5·0)		—	
		500	45·0 (±2·6)		43·5 (±2·3)		42·0 (±2·9)		—	

Table 6. *Influence of substrate upon the $q_{O_2}^{O_2}$ of normal and rheumatoid synovial tissues*

Figures in parentheses are the number of determinations. Medium, Krebs–Ringer-phosphate. Temperature, 37°. Gas phase, O_2.

Substrate	Substrate concn. (mM)	$q_{O_2}^{O_2}$ 'Normal' synovial tissue	Rheumatoid synovial membrane	Rheumatoid synovial villi
Malate	0	<1·0 (2)	2·1 (2)	—
	10	7·5 (2)	3·7 (2)	—
Fumarate	0	<1·0 (1)	2·0 (2)	22·0 (3)
	10	5·0 (1)	2·6 (2)	28·0 (3)
Succinate	0	<1·0 (4)	2·0 (2)	25·0 (5)
	10	6·0 (4)	4·3 (2)	40·0 (5)
Acetate	0	3·0 (1)	—	21·0 (2)
	10	3·0 (1)	—	21·0 (2)
Pyruvate	0	<1·0 (1)	2·0 (2)	24·5 (4)
	10	<1·0 (1)	3·7 (2)	23·0 (4)
Glucose	0	<1·0 (6)	1·7 (3)	30·0 (5)
	10	<1·0 (6)	1·2 (3)	31·9 (5)

Table 7. *Influence of hydrocortisone on the substrate metabolism of a rheumatoid synovium*

Substrates were used at a concentration of 10 mM. Medium, Krebs–Ringer-phosphate. Gas phase, O_2. Temperature, 37°.

Substrate	$q_{O_2}^{O_2}$ Control	250 µg. of hydrocortisone/ml.	Inhibition (%)
None	16·9	9·2	46
Acetate	16·4	7·6	54
Oxalacetate	28·8	12·7	55
cis-Aconitate	18·7	10·8	42
Citrate	18·2	10·2	44
α-Oxoglutarate	20·0	11·4	43
Succinate	18·8	11·5	39
Fumarate	25·1	12·0	52
Malate	18·0	10·3	43

of malate, fumarate and succinate markedly stimulated respiration. The rate of oxygen uptake by both rheumatoid synovial membrane and villi showed a similar stimulation by malate, fumarate and succinate, but not by acetate, pyruvate or glucose. As different tissues were used with each substrate to obtain the values given in Table 6, it was not possible to compare the relative oxidative stimulation by each substrate. We therefore tested the effect of various members of the tricarboxylic acid cycle on a single rheumatoid tissue. These results are given in Table 7. Acetate again had no stimulatory effect on $q_{O_2}^{O_2}$, though all the other substrates stimulated oxygen uptake; the most active in this instance were oxalacetate and fumarate. During this experiment hydrocortisone was introduced from the side arm after 46 min. and the rate of oxygen uptake measured for a further 60 min. The percentage inhibition of oxygen uptake, in the presence of hydrocortisone, was of roughly the same order for all flasks. There was no fall in respiration in the absence of hydrocortisone.

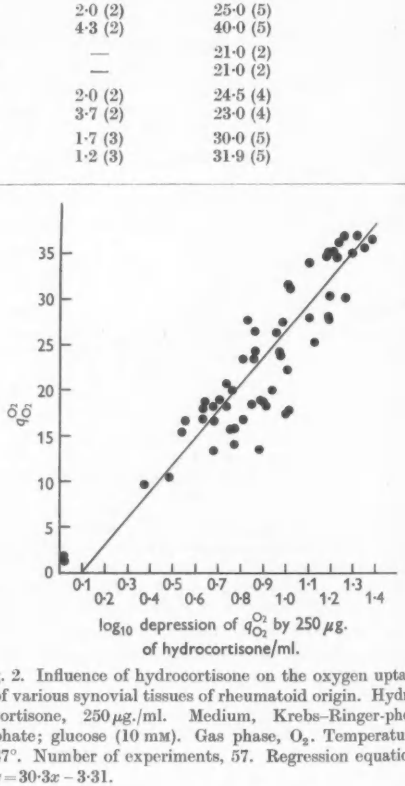

Fig. 2. Influence of hydrocortisone on the oxygen uptake of various synovial tissues of rheumatoid origin. Hydrocortisone, 250 µg./ml. Medium, Krebs–Ringer-phosphate; glucose (10 mM). Gas phase, O_2. Temperature, 37°. Number of experiments, 57. Regression equation, $y = 30{\cdot}3x - 3{\cdot}31$.

We have previously shown that the $q_{O_2}^{O_2}$ of rheumatoid synovia varied greatly, and that the oxidative metabolism might be correlated with the proliferative state of the tissue.

Fig. 2 shows a scatter diagram of the rates of oxygen uptake from a variety of rheumatoid tissues, plotted against the log of the depression of $q_{O_2}^{O_2}$

produced in that tissue by 250 μg. of hydrocortisone/ml. The values given in the diagram have accumulated over a period of 3 years, and in consequence the experimental technique with regard to the addition of hydrocortisone was not uniform. In one-third of the cases hydrocortisone was tipped from the side arm during incubation and the rate measured. In the remaining two-thirds hydrocortisone was present in the flasks during the whole experimental period, and an equal number of control flasks were put up. The pairs of observations in this latter group were randomly selected from all the observations on each tissue. The 'true-pair' regression line for $q_{O_2}^{O_2}$ on log depression $q_{O_2}^{O_2}$ was $y = 29{\cdot}65x - 3{\cdot}89$ for 21 pairs. The regression line for the 'random-pair' experiments was $y = 29{\cdot}23x - 3{\cdot}29$ for 36 pairs. There is not a statistically significant difference between these slopes and we have therefore treated all observations as if the experimental method was uniform. The resulting regression equation is $y = 30{\cdot}3x - 3{\cdot}31$. The correlation coefficient is 0·538 ($p < 0{\cdot}1\%$).

The same level of hormone gave increased inhibition with increasing synovial oxidative metabolism. The level of hydrocortisone used in these experiments (250 μg./ml.) is well within the range expected in the synovial fluid after intra-articular injection.

DISCUSSION

Intact synovial tissue was able to utilize a number of glycolytic intermediates *in vitro*. It was also capable of glycogen synthesis or breakdown. Glycogenolysis was increased in the presence of hydrocortisone, while aerobic and anaerobic glucose utilization and lactate production were markedly inhibited by the hormone. These inhibitory effects of this corticosteroid have been demonstrated on a number of other tissues [Bacila & Barron (1954), Miller (1954) and Martin, Chaudhuri, Green & McKinney (1954)].

After homogenization of synovial tissue it was necessary to make a number of additions to the medium to achieve even a moderate rate of glycolysis. The most important of these additions appeared to be DPN. The increased stabilization of the glycolytic rate in the presence of DPN and nicotinamide was presumably due to inhibition of breakdown of DPN. Under these conditions no inhibition by hydrocortisone of acid production could be observed. Splenic homogenates, however, showed rapid rates of glycolysis without the addition of cofactors and were inhibited by hydrocortisone. When the splenic homogenates were fortified, the degree of inhibition was often reduced, and was apparent only at a later period of incubation. Thus it would appear that the inhibitory action of hydrocortisone may be modified by the presence of various glycolytic intermediates and cofactors. The marked stimulatory effect of DPN upon the glycolysis of synovial homogenates suggests that the synovial content of this coenzyme may be low. Branster & Morton (1956) have suggested that a decreased rate of synthesis of DPN may be one aspect of rapid cell proliferation. Jedeikin & Weinhouse (1955) have reported that the total DPN contents of a variety of transplanted solid and ascites tumours were considerably lower than those of normal tissues. As the rheumatoid synovia used in our experiments were in an active state of proliferation it would be of interest to know the level of the tissue DPN. Our findings with suspension of rat spleen appear to indicate that the inhibitory action of the hormone can be demonstrated in broken-cell preparations. These results are at variance with the views expressed by Thorn *et al.* (1953), who stated that no effect of cortisone on enzyme systems had been clearly demonstrated in the absence of the intact cell, and with the findings of Martin *et al.* (1954), who showed that the inhibitory effect of cortisone and hydrocortisone on aerobic lactate formation was seen only in intact human leucocytes, which had been handled with care to prevent injury.

Our results indicate that various intermediates of the tricarboxylic acid cycle can be readily oxidized, and that their respective enzyme systems can be presumed to be present. The question of the complete operation of the citric acid cycle in normal and rheumatoid synovia is of considerable importance, as it has been shown that the presence of rheumatoid disease in the synovial membrane is accompanied by a very considerable increase in oxidative metabolism, e.g. $<1{\cdot}0$ up to $q_{O_2}^{O_2}$ 37·0 (Dingle & Thomas, 1956). This minimal oxidative rate found in normal synovia might be due to a very low level of oxidative enzymes, or an incomplete operation of the tricarboxylic acid cycle, or to the effect of some rate-controlling factor. If the presence of rheumatoid disease can be presumed to alter any of these conditions, the oxidative level of the tissue might be raised. As the addition of malate, fumarate, and succinate raised the $q_{O_2}^{O_2}$ of some normal tissue to a value approaching that of some rheumatoid tissues, it is unlikely that the minimal synovial respiration normally present is due to a low level of oxidative enzymes. Whether or not the Krebs's cycle is fully operative in both normal and rheumatoid synovia, however, must await further investigation. The inhibition of oxygen uptake by hydrocortisone appeared relatively constant regardless of the added substrates, which minimizes the possibility of inhibition of a specific enzyme system within the cycle.

In view of its inhibitory action on both glycolysis and oxidation, it is possible that the action of

hydrocortisone is upon some form of metabolic pacemaker.

It has previously been shown that the $q_{O_2}^{O_2}$ of rheumatoid synovia is increased in proliferating tissues. Fig. 2 indicates that the inhibitory effect of the hormone is greatest in the most metabolically active synovia. It is likely therefore that the inhibition of oxidation and glycolysis must result in a marked deprivation of energy to the most actively proliferating tissues. This is substantiated somewhat by clinical experience of intra-articular injections of hydrocortisone, which appear to be most effective when the proliferative phase is most marked. It is of interest in this connexion that Roberts & Szego (1953) found that the initiation of true growth in the rat uterus, occurring 20 hr. after administration of oestradiol, was associated with a substantial increase in respiration as well as in glucose utilization, and that hydrocortisone was effective in inhibiting these metabolic changes.

SUMMARY

1. The effect of various substrates on the metabolism of normal and rheumatoid synovial tissues *in vitro* has been investigated in the presence and absence of hydrocortisone.

2. Rheumatoid synovial membrane has been shown to be capable of glycogen synthesis or breakdown. The presence of hydrocortisone resulted in an increase in glycogenolysis or a diminution of glycogen synthesis where this occurred.

3. Glucose utilization of synovial tissue is inhibited by hydrocortisone under both aerobic and anaerobic conditions.

4. Rheumatoid synovial tissue utilized glucose 1-phosphate, glucose 6-phosphate and fructose 1:6-diphosphate under aerobic conditions. The utilization of these substrates gave variable results in the presence of hydrocortisone, though the inhibitory action on $q_{O_2}^{O_2}$ was unaffected.

5. Aerobic and anaerobic lactic acid formation was inhibited in the presence of hydrocortisone.

6. The effect on the $q_{O_2}^{O_2}$ of normal and rheumatoid synovia by malate, fumarate, succinate, acetate, pyruvate and glucose has been investigated. Malate, fumarate and succinate stimulated oxygen uptake. None of these substrates affected the respiratory inhibition produced by hydrocortisone.

7. The inhibitory effect of a standard hydrocortisone concentration, on $q_{O_2}^{O_2}$ of rheumatoid synovial tissues of varying degrees of metabolic activity, was greatest in those tissues which had the highest rates of oxygen uptake.

8. Synovial homogenates showed only slight anaerobic acid production unless diphosphopyridine nucleotide was added. With optimum conditions for glycolysis, hydrocortisone showed no inhibitory action.

9. Splenic homogenates showed significant inhibition of anaerobic acid production by hydrocortisone.

We should like to thank Dr G. D. Kersley for his interest and encouragement; the orthopaedic surgeons of the Bath clinical area for the tissue specimens and Dr G. Herdan for his advice on statistical analysis. The hydrocortisone suspension was generously supplied by the Medical Research Council. Thanks are also due to Miss A. Langley for technical assistance.

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A Colorimetric Method for the Estimation of Tyrosine

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A considerable need exists for a method of estimating tyrosine. The method first described by Gerngross, Voss & Herfeld (1933), which depends on the reaction between tyrosine and 1-nitroso-2-naphthol in the presence of an oxidizing agent (usually nitric acid), appears to meet the requirements. The red colour which is the result of the reaction is, however, destroyed by light, and neither the original method nor a later adaptation by Thomas (1944) overcomes this disadvantage. The colour fades sufficiently rapidly for the reaction to be unsuitable as a routine method. Maciag & Schoental (1938) were able to stabilize the colour by adding iron alum to the solution, but this reduced the sensitivity of the method considerably; the conditions which they describe are also not the best for accurate work. Udenfriend & Cooper (1952) allowed the red-coloured compound to be further oxidized by nitric acid to a stable yellow pigment, excess of nitrosonaphthol then being extracted with a water-immiscible solvent. A considerable part of the reaction product from tyrosine is extracted by the solvent, and the pigment formed from compounds not containing an amino group, such as *p*-hydroxyphenylpyruvic acid, is completely soluble in the solvent and hence cannot be estimated. This method is more complicated than that to be described.

In view of the simplicity and sensitivity of the Gerngross reaction, factors controlling the intensity of colour development, and the stability of the colour, have been investigated. As a result a method for the estimation of tyrosine has been developed, which can be used for the analysis of solutions containing as little as 1 μg./ml. A preliminary report of the method has been made (Ottaway, 1957).

Ceriotti & Spandrio (1957), who have very courteously allowed me to see their manuscript after the present paper had been submitted for publication, have also re-examined the nitrosonaphthol reaction. Apart from the method of stabilizing the colour, the conclusions reached by Ceriotti & Spandrio with regard to reagent concentrations and general conditions were very similar to those described here.

MATERIALS AND METHOD

L-Tyrosine was obtained from Roche Products Ltd. (Welwyn Garden City, Herts.). 1-Nitroso-2-naphthol was AnalaR grade; it was not recrystallized before use, but solutions in ethanol or acetone were filtered before making up to volume. Protein hydrolysates were Hydrolysed Casein, Allen and Hanbury Ltd., and Casein Hydrolysate, B.D.H. Ltd.

For general work an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) was used. The absorption spectrum of the reaction product was determined with a Unicam SP. 600 spectrophotometer (Cambridge Instrument Co.).

The method of colour production used in the experiments described in this paper was as follows. The nitrosonaphthol reagent was added to 4 ml. of the solution containing tyrosine in a test tube (6 in. $\times \frac{5}{8}$ in.). Conc. HNO_3 was added from a dropping pipette so that the drops fell through the solution and did not touch the walls of the tube. The solution was then placed in a water bath at a selected temperature, care being taken not to agitate the tube and so disturb the HNO_3 at the bottom. At the end of a selected time, the solution, which now showed a red ring at the bottom, was swirled vigorously and returned to the bath for a further time, usually the same as the first, to complete colour development. This procedure was found to give very satisfactory results. The directions of Maciag & Schoental (1938), which are described under 'Recommended procedure', are simpler and give equally good results.

RESULTS

Factors controlling development of colour

Temperature. In Fig. 1 are shown the effects on colour intensity of varying the temperature to which the solution was heated, and also the total length of time for which it was incubated. The colour is destroyed by heating with HNO_3, particularly at 100°. Thus although colour development was very quick when the tubes were heated in a boiling-water bath, full colour was never obtained, nor was it possible to get reproducible results. At temperatures lower than 60°, colour development was very slow; at 40° it required more than 1 hr. After incubating at 100° and 80°, more of the colour was preserved if the tubes were cooled quickly in cold water (see Fig. 1), but at 60° the difference between air-cooling and water-cooling was very slight.

Concentration of acid. Colour intensity increases with nitric acid concentration up to a maximum value. For example, with 100 μg. of tyrosine per tube, optical density rose from 0·015 with 1 drop of conc. HNO_3 to 0·435 with 5 drops; 6 drops per tube produced an optical density of 0·410. Since a large excess of acid destroyed the colour quite rapidly, the minimum volume required for full colour development was chosen. This was about 1 drop (0·022 ml.) of conc. HNO_3/ml. of tyrosine solution, which means that the solution after mixing was approx. 0·35 M with respect to HNO_3.

The effect of acidifying the solution with acids other than nitric, before colour development, was tested. Mineral acids were found to be without effect up to M-concentrations (cf. Ceriotti & Spandrio, 1957). Some complex acids, in high concentrations, inhibited colour development, and these are discussed in the section on 'Interfering substances'.

Ethanol. Ethanol was found to reduce the colour intensity considerably. It may inhibit colour development, but it is probable that it also causes the colour to fade more rapidly, as the following experiment shows. Colour was developed in tubes containing 100 μg. of tyrosine by the addition of 0·03 ml. of 1 % nitrosonaphthol in ethanol, and to the cooled solutions were added varied volumes of ethanol, with sufficient water to make the total volume up to 8 ml. The rate of colour fading ranged from 21 %/hr. with no added ethanol, to 100 % with 2 ml. of added ethanol (see also Fig. 3).

Ethanol and other alcohols were judged to be unsuitable solvents for the nitrosonaphthol reagent. Acetone impedes colour development to a much less extent than ethanol and, if added after development was complete, was found not to increase the rate of fading at all.

Concentration of nitrosonaphthol. The effect on colour intensity of varying the amount of nitrosonaphthol reagent added to 4 ml. of tyrosine solution is shown in Table 1. The optimum amount of reagent varied according to the amount of tyrosine to be estimated; with 250 μg. of tyrosine, maximum colour development required the addition of 1000 μg. of nitrosonaphthol. Even with this amount of reagent, Beer's Law was never obeyed as Table 1 shows, and a great disadvantage was that a white haze formed in the solutions on cooling. This was evidently some insoluble oxidation product of the reagent (since it was most intense in the blank), although its colour showed that it was not nitronaphthol. It did not form if less than 250 μg. of nitrosonaphthol was added to 4 ml. of tyrosine sample. This was hardly enough to ensure adequate sensitivity, so the quantity finally decided on was 300 μg./sample.

The haze which formed when this quantity of reagent was used was a very finely divided precipitate and not easy to centrifuge down. It was found more convenient to remove it by adding a suitable solvent, such as acetone, while diluting the solutions to a convenient volume for the colorimeter.

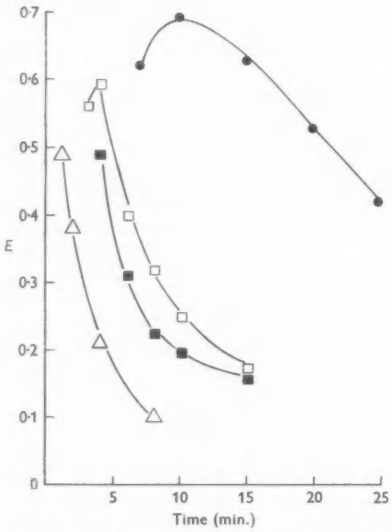

Fig. 1. Effect of temperature and heating time on colour formation and extinction. ●, 60°; □, 80°, water-cooled; ■, 80°, air-cooled; △, 100°.

Table 1. *Effect of nitrosonaphthol concentration on colour intensity*

Volume of solution in each tube was 4 ml. The nitrosonaphthol added was dissolved in 0·1 ml. of acetone. After the addition of 4 drops of conc. HNO_3 to each tube, colour development was carried out for 12 min. at 60°. Each tube was read in the colorimeter against a blank containing the same amount of nitrosonaphthol. Figures represent optical densities.

	Optical density					
Nitrosonaphthol added (mg.) ...	0·1	0·2	0·3	0·4	0·7	1·0
Tyrosine/tube (μg.)						
100	0·390	0·428	0·496	0·498	0·502	0·492
250	0·568	0·744	0·790	—	0·880	0·900

Table 2. *Effect of inorganic salts on colour stability*

Each tube contained 100 μg. of tyrosine and colour development was carried out for 12 min. at 60°. The salts were introduced either before or after colour development, by the addition of varying volumes of M-salt solution. The total volume was made up to 4 ml. before development, or 8 ml. after. Figures represent the optical densities when the samples were read against the appropriate blanks, and the values in the first half of the Table are the means of six observations.

Time after completion of colour development (min.)	Optical density 10	20	40	60	120	Percentage drop in intensity, 20–60 min.
Addition before or after development						
None	0·420	0·396	0·372	0·344	—	13
1 ml. of NaCl; after	0·414	0·402	0·400	0·392	0·368	2
1 ml. of NaCl, 1 drop of $FeCl_3$; after	0·422	0·414	0·414	0·406	0·390	2
1 ml. of NaCl; before	0·470	0·450	0·450	0·440	—	2
2 ml. of NaCl; before	0·440	0·430	0·424	0·410	—	4·5
3 ml. of NaCl; before	0·380	0·390	0·362	0·348	—	4·5
0·5 ml. of Na_2SO_4; before	0·494	0·460	0·440	0·420	—	8·5
1 ml. of Na_2SO_4; before	0·340	0·320	0·312	0·300	—	6

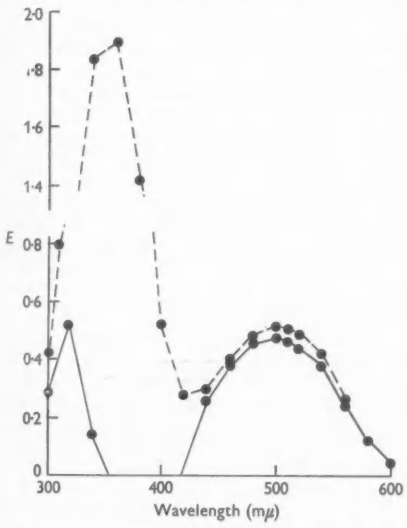

Fig. 2. Absorption spectrum of pigment, developed from 100 μg. of tyrosine and 300 μg. of 1-nitroso-2-naphthol in a final volume of 8 ml. The blank contained nitroso-naphthol: stabilized with acetone and NaCl (continuous line); stabilized with acetone, NaCl and $FeCl_3$ (broken line).

Characteristics and stability of the colour

The red pigment obtained during the reaction has the absorption spectrum shown in Fig. 2 (full line). There is a sharp peak with a maximum at 320 mμ and a broader peak with a maximum at 500 mμ. The latter band was used for the estimation of tyrosine. Beer's Law was obeyed only in the range 0–80 μg. of tyrosine, even with monochromatic light (see also Maciag & Schoental, 1938).

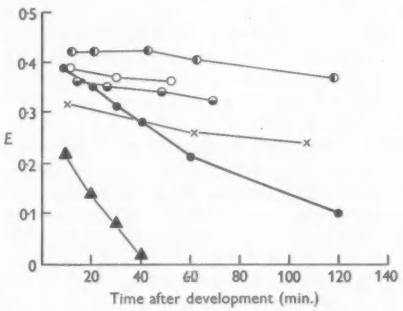

Fig. 3. Effect of various substances on the stability of the red colour developed by 100 μg. of tyrosine. Each addition was made after colour development was complete. ●, Acetone; ○, acetone, tubes kept in dark; ◒, acetone, 0·125 M-NaCl; ◐, acetone, 0·125 M-NaCl, 2 drops of $FeCl_3$ soln.; ×, acetone, 1% potassium ferricyanide; ▲, 25% ethanol.

The pigment is destroyed by light at a rate sufficiently fast to make quantitative application of the method difficult (Table 2 and Fig. 3). In the conditions used here the loss of intensity is about 20%/hr. In the dark the colour is quite stable (Fig. 3). Gerngross *et al.* (1933) and Thomas (1944) describe procedures which make allowance for the fading colour, but neither is very suitable for routine use. It seemed likely that destruction of the pigment was due to light absorbed in the band centred at 320 mμ, and various compounds with a strong absorption in this region were tried as stabilizers. The addition, after colour development, of an equal volume of 1% potassium ferricyanide was found to be effective, but it absorbed enough light at 500 mμ to reduce the sensitivity of the method considerably. The same objection applies to the addition of an equal volume of saturated

ferric ammonium sulphate, which was recommended by Maciag & Schoental.

It was observed by chance that the colour faded much less rapidly if the solutions to be analysed contained NaCl. Further investigation showed that the addition to the solution, after colour development, of an equal volume of 0·5 M-NaCl was as effective in stabilizing the pigment as ferricyanide. No reason was found why this should be so. It was observed that the salt was slightly more effective if added before colour development, although concentrations above M diminished the colour, and that the effect was temporary, as slow fading began about an hour after adding the salt solution. Sodium chloride was found to be very much more effective than an equimolar concentration of Na_2SO_4. This last observation appeared to rule out the possibility that stabilization was a result of reducing the concentration of dissolved oxygen. It is noteworthy that Ceriotti & Spandrio (1957) were able to produce a stable pigment by carrying out the reaction in approx. 2 N-HCl. It is possible that this is due to the presence of Cl^- ions, rather than to pH.

The addition, after colour development, of a few drops of 0·5 M-$FeCl_3$ appeared to give stabilization for a rather longer period than NaCl alone. Ferric chloride by itself was ineffective at this concentration. The intense absorption of what is presumably the undissociated Fe_2Cl_6 molecule in the region of 300 mμ is shown in Fig. 2 (broken line).

Recommended procedure

In the light of the observations described, the following procedure is suggested for the estimation of tyrosine in solutions which do not contain interfering substances (for a discussion of these, see below).

Reagents. 0·3% 1-Nitroso-2-naphthol in acetone: the solution is filtered before making up to volume. It is stable indefinitely, but must be protected from evaporation.

Concentrated nitric acid.

Stabilizing reagent: the precise composition of this solution may be varied, according to the final volume of sample which is required. For the volumes described below, the solution was made up as follows. To 50 ml. of M-NaCl was added 75 ml. of acetone, and the mixture was made up to 300 ml.; 5 ml. of 0·5 M-$FeCl_3$ was added immediately before use. (A precipitate, probably of the double salt of $FeCl_3$ and NaCl, develops if the complete solution is allowed to stand overnight.)

Standard tyrosine solution: 25 mg. of tyrosine is dissolved in a few millilitres of boiling water, and this solution is quickly diluted to 100 ml. It is stable for several weeks if kept in the refrigerator.

Procedure. To 2 ml. of solution, containing 10–200 μg. of tyrosine, is added 0·1 ml. of nitrosonaphthol reagent. The solutions are mixed and placed in a water bath at 60° for 5 min., or until temperature equilibrium has been reached. The solution is then removed from the bath and 4 drops of conc. HNO_3 are added from a dropping pipette. Since, as shown above, the amount of nitric acid added affects the amount of colour developed, it is as well always to use the same pipette. Colour development starts almost immediately. The solution is returned to the water bath for 1 min. It is then removed, mixed by swirling, and allowed to cool in air for about 5 min., after which the stabilizing reagent is added. A volume (6 ml.) of the stabilizing reagent described above was usually added to the cooled solution, but the composition of this reagent and the volume to be added may be altered as convenient, so long as the final solution contains 0·12 M-NaCl and 25% of acetone. Colour intensity is best read within 10–45 min. after adding the stabilizing reagent, with an OB2 (blue) filter (Evans Electroselenium Ltd.). A reagent blank and suitable standards, prepared by diluting the stock tyrosine solution, are run at the same time.

Interfering substances

Fe^{2+}, Co^{2+} and Ni^{2+} ions interfere by forming coloured complexes with nitrosonaphthol. Fe^{3+} ions do not seriously interfere unless they are present in high concentrations, when their effect may be lessened by acidifying the solution with dilute HCl before adding the colour reagent. Cu^{2+} ions in low concentrations (below 0·01 M) do not interfere.

Of the protein-precipitating reagents, tungstic and molybdic acids interfere seriously, particularly if phosphate is also present. The effect of ethanol has been described above. Trichloroacetic acid is without effect on the reaction.

Strong reducing agents, such as cysteine or ascorbic acid, inhibit colour development. They are best removed by warming the solution to 60° with a drop of conc. HNO_3 before adding the colour reagent. Glucose does not interfere in concentrations up to 0·02 mg./ml., if colour development is

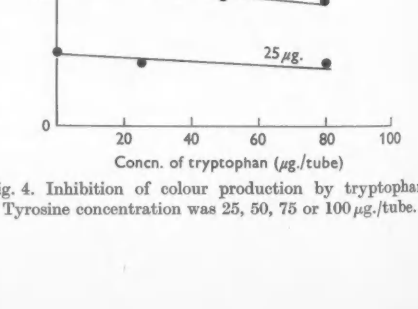

Fig. 4. Inhibition of colour production by tryptophan. Tyrosine concentration was 25, 50, 75 or 100 μg./tube.

Table 3. *Reproducibility of the reaction in protein hydrolysate solutions*

Regression coefficients were calculated from the data below for the following solutions: (1) pure tyrosine in water; (2) a 3 mg./ml. solution of a casein hydrolysate containing about 0·1 % of tyrosine, to which were added amounts of tyrosine varying from 5 to 20 μg./ml.; (3) a solution of a casein hydrolysate estimated to contain 1·65 % of tyrosine. Colour development was carried out in the standard way; the total volume in each tube before development was 4 ml. Figures are the means of four replicates at each concentration.

(1) Standard tyrosine solution

Tyrosine concn. (x) (μg./ml.)	5	10	15	20	25
Optical density (y)	0·088	0·180	0·246	0·316	0·396

(2) Low-tyrosine casein hydrolysate

Added tyrosine concn. (x) (μg./ml.)	0	5	10	15	20
Optical density (y)	0·052	0·150	0·236	0·304	0·386

(3) High-tyrosine casein hydrolysate

Estimated tyrosine concn. (x) (μg./ml.)	4·25	8·5	12·75	17	21·25
Optical density (y)	0·062	0·142	0·210	0·282	0·340

Regression coefficients, b, were calculated for the equations $y = a + b(x - \bar{x})$ by the method of least squares.

	Regression coefficient (b)	Variance of b
Solution 1	0·375	0·00064
Solution 2	0·412	0·00014
Solution 3	0·371	0·00099

t for $b_1 - b_2 = 1·32$, t for $b_1 - b_3 = 0·1$ } i.e. neither difference is significantly different from zero

carried out at 60°. Above this concentration there is a slight inhibition, amounting to 6 % at 0·25 mg. of glucose/ml.

Tryptophan. This compound does not itself give a colour in the conditions described here, but it inhibits colour development to an extent depending on its concentration. The percentage inhibition of colour development is independent of tyrosine concentration (Fig. 4) so that it is possible to use internal standards. This precaution is probably not necessary for general work, since the inhibition of colour development is very small when the tryptophan in a solution is less than 5 μg./ml. As tryptophan amounts to less than 2 % of most proteins, the use of internal standards may be neglected when analysing protein hydrolysates containing less than 25 mg. of total amino acids/ml.

Specificity

A list of many *p*-alkylated phenols which had been found to give a colour with 1-nitroso-2-naphthol was given by Gerngross *et al.* in their original paper. These authors also gave a list of related compounds which they had found not to react. These lists have not been generally checked, but a certain number of compounds, mainly of biological interest, have been tested in the present method. Adrenalin, thyroxin, 3:4-dihydroxyphenylalanine, 3:5-dihydroxyphenylpyruvic acid, homogentisic acid and *p*-hydroxybenzoic acid did not react. Tyrosine, *p*-hydroxyphenylpyruvic acid, glycyltyrosine and *p*-cresol gave the characteristic red colour. Glycyltyrosine gave a more intense colour than an equivalent concentration of free tyrosine. Tyrosine in combination in proteins (except alkali-denatured protein) also reacts, but the use of this method for estimating tyrosine in unhydrolysed proteins is limited to those proteins which do not precipitate in dilute HNO_3 solutions. The impression was gained that tyrosine contained in proteins does not give such an intense colour as the free amino-acid. *o*- and *m*-Tyrosine give a brown pigment, which is easily distinguished from that given by *p*-tyrosine. The extinction of the brown pigment is only one-third of that of the red tyrosine pigment when measured at 500 mμ.

Sensitivity and reproducibility

Dilution of the nitrosonaphthol reagent tenfold, in order to minimize the yellow masking colour, enabled the presence of 1 μg. of tyrosine/ml. to be detected in a solution. This limit of sensitivity is not suitable for quantitative use at this concentration, however; the lower limit for quantitative work is about 10 μg./tube. No upper limit for tyrosine concentration was found, but above 200 μg./ml. the curve relating concentration to colour intensity begins to flatten considerably, so that the sensitivity of the method is much less in this range. This is so even if the reagent concentration is increased. The standard deviation of replicate estimations over the range 25–200 μg./tube was 2·6 % of the mean.

The reproducibility of the method in the analysis of more complex solutions was tested by comparing the slope of the line relating optical density to concentration of tyrosine in water and in casein hydrolysate solutions (Table 3); and by comparing

Table 4. *Recovery of tyrosine*

Colour was developed in the standard way in a solution containing 1·6 mg. of casein hydrolysate/ml. The extinction was measured and the solution was then diluted to exactly twice the original volume. Tyrosine concentration of the dilute solution was estimated from the standard curve, and sufficient tyrosine was added to it to double its estimated concentration. Colour was developed in the new solution, and its extinction was measured. Figures are the means of six replicates for each solution, with standard deviations.

	Extinction	Tyrosine concn. (μg./ml.)
(*a*) Original solution	0·378±0·028	—
(*b*) Diluted solution	0·198±0·022	12·1
(*c*) Diluted solution with 12·1 μg. of added tyrosine/ml.	0·372±0·028	—

t for (*a*) − (*c*) = 0·46. Not significant.

the optical density produced by the same concentration of tyrosine in different concentrations of protein hydrolysate (Table 4).

Applications

This reaction has been investigated particularly because of its convenience in the study of tyrosine metabolism. As it measures only tyrosine and *p*-hydroxyphenylpyruvic acid, it gives very good agreement between tyrosine disappearance and oxygen consumption in the rat-liver tyrosine oxidase system described by Knox & LeMay-Knox (1951). The method can be easily applied to the estimation of tyrosine in protein hydrolysates, if precautions are taken against interference by tryptophan. Similar precautions must be taken in the estimation of tyrosine in urine, since urine contains sufficient tryptophan derivatives to inhibit colour development seriously. If the urine is diluted 10- to 20-fold, and internal standards are used in addition, the estimation can be performed without difficulty. An estimation performed on a 24 hr. sample of normal urine by this method gave a figure of 72 mg. of excreted tyrosine/day. This is just within the range of 46–72 mg./day for total (free + conjugated) tyrosine excretion reported by Ulrich, Schropp & Martin (1954), who used a microbiological method of estimation. The use of the present method for the determination of tyrosine in blood appears feasible. Hier & Bergeim (1946) report a value of 15 μg./ml. for free tyrosine in human plasma. Estimations of tyrosine in deproteinized human plasma, trichloroacetic acid being used as suggested by Udenfriend & Cooper (1952), give reasonable values of about 15–20 μg. of tyrosine/ml. of plasma. In one instance a plasma ultrafiltrate was prepared and found to contain the same concentration of tyrosine as the same plasma when deproteinized by trichloroacetic acid.

SUMMARY

1. A simple and reliable method of estimating tyrosine has been developed, based on earlier work, which may be used for the determination of 10–250 μg. of tyrosine in 2–4 ml. samples.

2. The effects of a number of interfering substances have been investigated, and precautions are described for the determination of tyrosine in various biological fluids.

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Latent Phenolase in Extracts of Broad-Bean (*Vicia faba* L.) Leaves

2. ACTIVATION BY ANIONIC WETTING AGENTS*

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Kenten (1957) has shown that water extracts of broad-bean leaves contain much latent phenolase. Active phenolase was released by brief exposure of the extracts to acidic (pH 3–3·5) or alkaline (pOH 2·5–3) conditions, or by incubating in the presence of ammonium sulphate at about pH 5. The nature of the latent phenolase was not elucidated but it was suggested that either a phenolase precursor or a phenolase–protein-inhibitor complex was present in the leaf extracts. The present work describes some further properties of the latent phenolase and shows that activation follows treatment with certain anionic wetting agents.

* Part 1: Kenten (1957).

MATERIALS AND METHODS

Estimation of phenolase activity. Phenolase activity was estimated either colorimetrically or manometrically. The manometric method is preferable when crude extracts are used since these have low transparency and are liable to contain reducing materials which prevent the accumulation of the phenolic oxidation product. The manometric method is also more suitable when the oxidation of monophenols is studied, first because the initial step in their oxidation involves *ortho*hydroxylation and so does not immediately give rise to coloured oxidation products, and secondly because the oxidation is preceded by a variable and sometimes prolonged lag period.

The colorimetric method has proved to be the most convenient one with clarified and dialysed leaf extracts. Here the rate of formation of the red initial oxidation product of dihydroxy-DL-phenylalanine (DOPA) is measured with an EEL (Evans Electroselenium Ltd.) colorimeter with a Bright-Spectrum Blue-Green Filter no. 623, maximum transmission at 495 mμ. The reaction mixtures consisted of 3 mg. of DOPA, with or without wetting agent, and dialysed water extract of broad-bean leaf in a total volume of 6 ml., containing 25 mM-citrate at pH 6. The dialysed water extract of broad-bean leaf was added 10–15 sec. before zero time and it was thoroughly mixed in at zero time. The first reading was taken at 20 sec., and readings thereafter at 30 sec. intervals. The increase in light absorption over the period 20–200 sec. was taken as a measure of the phenolase activity. With this method there is a linear relationship between the amount of liberated phenolase and the rate of formation of the oxidation product of DOPA over a wide range of enzyme concentration (Fig. 1).

Manometric reaction mixtures in a total volume of 3 ml. at 25° consisted of 3 mg. of DOPA or 3 mg. of *p*-cresol in 50 mM-citrate at pH 6, with or without wetting agent, in the main vessel. After equilibration, dialysed water extract of broad-bean leaf was added from the side arm. Potassium hydroxide was present in the centre well, the gas phase was air and the temperature 25°.

Plant material and extraction procedure. The broad-bean leaves used were taken from young plants grown in John Innes Compost in a heated greenhouse. The leaves were ground with sand and an equal weight of water previously cooled to 0° in a chilled mortar. The slurry was squeezed by hand through strong cotton cloth, the extract being collected in a cylinder surrounded by ice and water. The extract was centrifuged in an MSE (Measuring and Scientific Equipment Ltd.) angle-head centrifuge at about 8000 *g* for 15 min. in a cold room at 0–4°. The supernatant was dialysed for 24 hr. at 0–4° against large volumes of buffer, pH 6–6·5, 4 mM with respect to both phosphate and citrate. The dialysed extract was then frozen and thawed twice, clarified by centrifuging at 8000 *g* for 15 min. and stored frozen at −18°. These dialysed extracts had only a trace of phenolase activity and could be stored frozen for at least a month without appreciable change either in phenolase activity or phenolase activity released by treatment with anionic wetting agents (latent phenolase). Table 1 shows the activities of phenolase and latent phenolase of some of the preparations used in the present work. Under the conditions of estimation of latent phenolase used in Table 1, the amount of liberated phenolase activity is not directly proportional to the O_2 uptake in the first 10 min. of the reaction (Fig. 1). However, the departure from linearity

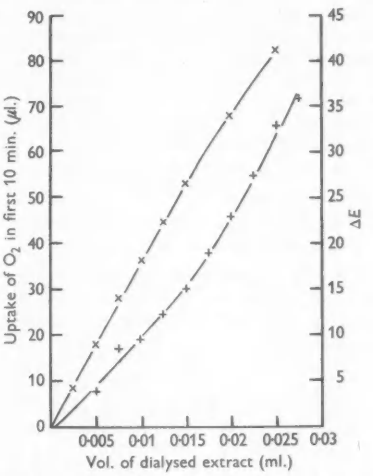

Fig. 1. Effect of varying the amount of dialysed water extract of broad-bean leaf on the oxidation of DOPA in the presence of 4 mM-Aerosol OT at pH 6. +, Manometric measurements of the O_2 uptake; ×, colorimetric measurements of the rate of formation of the oxidation product of DOPA at 21°.

Table 1. *Phenolase and latent phenolase in dialysed water extracts of broad-bean leaf*

Phenolase activity was estimated manometrically with DOPA as substrate in the absence and in the presence of 4 mM-Aerosol OT. The values are corrected for the blank uptakes with heated extract (5 min. at 100°). These blanks were less than 3 μl. of O_2/10 min.

Dialysed water extract preparation	Total N (mg./ml.)	O_2 uptake in first 10 min. (μl.): Phenolase (0·6 ml. of extract in the absence of Aerosol)	O_2 uptake in first 10 min. (μl.): Latent phenolase (0·02 ml. of extract in the presence of Aerosol)	Latent phenolase (μl. of O_2/mg. of N/hr. in the presence of Aerosol)
A	0·54	8	30·5	17 100
B	0·71	0	27·0	10 400
C	1·29	0	48·5	11 260
D	0·84	9	58·5	20 900

is not serious and it is considered preferable to express these results in quantitative terms of one of the reactants rather than in the relative colorimetric units. The relationship between the rate of oxidation and amount of enzyme obtained in the manometric experiments of Fig. 1 is similar to that obtained by Adams & Nelson (1938) in manometric studies of mushroom phenolase. Their evidence suggests that this type of relationship may result from a protective action of the protein contained in the preparation on the activity of the phenolase.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 and KOH, citrate buffers from solutions of citric acid and sodium citrate, pyrophosphate buffers from solutions of $K_4P_2O_7$ and $Na_2H_2P_2O_7$.

Measurements of pH. These were made with a glass electrode.

Wetting agents. The commercial wetting agents used were Triton X 100 (an alkyl aryl polyether alcohol, Rohm and Haas Co.) and Lissapol N (an ethylene oxide condensate with substituted phenol or fatty acid, Imperial Chemical Industries Ltd.), both non-ionic, and Aerosol OT 100% (sodium dioctylsulphosuccinate, Hardman and Holden Ltd.), which is anionic. For convenience in plotting on a molar basis and comparison with the other wetting agents, it has been assumed that the last-named compound was pure sodium dioctylsulphosuccinate.

RESULTS

Activation of latent phenolase by treatment with anionic wetting agents

Effect of the nature and concentration of wetting agent. The effect of the presence of different wetting agents on the phenolase activity of dialysed water extracts of broad-bean leaf has been studied manometrically and colorimetrically.

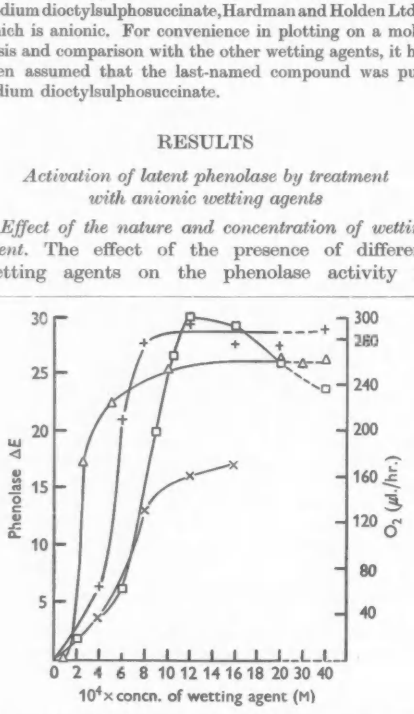

Fig. 2. Effect of different anionic wetting agents on the phenolase activity of dialysed water extract of broad-bean leaf at pH 6. △, Aerosol OT, *p*-cresol as substrate, 0·1 ml. of dialysed extract, activity measured manometrically; +, Aerosol OT; □, sodium dodecyl sulphate; ×, sodium oleate, activity measured colorimetrically with DOPA as substrate at 19–20° with 0·014 ml. of dialysed extract.

The manometric experiments were made with *p*-cresol as substrate and varying amounts of Aerosol OT. With *p*-cresol the O_2 uptake began after a lag period which was variable and sometimes as long as 30 min. In the second hour the rate of O_2 uptake remained practically constant, and it is from measurements in this period that the activities in Fig. 2 have been calculated. The autocatalytic nature of the reaction with monophenol but not with *o*-dihydric phenol is typical of the behaviour of other plant phenolases (e.g. Dawson & Tarpley, 1951).

The results obtained with some of the anionic agents (Fig. 2) show that the phenolase activity increases as their concentration is increased until a maximum is reached; further increase in wetting agent is then either without effect (Aerosol OT) or reduces the activation (sodium dodecyl sulphate). Whereas the shapes of the curves given in Fig. 2 were reproducible, the position relative to the abscissa varied somewhat in different experiments. The maximum activity obtained with sodium dodecyl sulphate (SDS) was about the same as that obtained with Aerosol OT. In the experiments with sodium oleate some oleic acid came out of solution at pH 6 and decreased the transparency of the solution. For this reason it was not possible to study the effects of high concentrations of sodium oleate by the colorimetric method.

A number of other wetting agents were tested under the conditions of Fig. 2 at 0·5, 3 and 20 mM, where the molecular weight was known, and at about 0·2, 1·3 and 8 g./l. where this was unknown. No activation was observed either with the cationic wetting agents cetyltrimethylammonium bromide (CTAB) and cetylpyridinium bromide, or with the nonionic agents saponin, Triton X 100 and Lissapol N.

When the dialysed extract was heated for 3 min. at 100° before testing, no phenolase activity was detected in the presence of the anionic wetting agents.

Effect of chain length on the activating capacity of sodium n*-alkyl sulphates.* The activating effect of a number of sodium *n*-alkyl sulphates has been studied with the results shown in Fig. 3. The experiments were made by the colorimetric method with DOPA as substrate; a single batch of dialysed water extract of broad-bean leaf was used throughout. No activation was found when the dialysed extract was heated for 5 min. at 100° before testing. When sodium butyl sulphate was tested under the conditions of Fig. 2 a slight activating effect was found at about M concentration. Tests showed that the presence of high concentrations of sodium butyl sulphate inhibited the phenolase activity

towards DOPA; at 2 M inhibition was complete, at M about 70 % whereas at 0·1 M there was no appreciable effect. These tests were made with a dialysed water extract, previously activated by treatment with Aerosol OT, as enzyme. Accordingly, the curve given in Fig. 3 was obtained by mixing the enzyme extract with sodium butyl sulphate before testing for phenolase activity. To 0·8 ml. of solution containing varying amounts of sodium butyl sulphate in 40 mM-citrate buffer, pH 6, 0·1 ml. of dialysed extract was added and immediately after mixing a 0·1 ml. sample was withdrawn and the phenolase activity estimated colorimetrically, as described previously, in a mixture containing 3 mg. of DOPA in a total volume of 6 ml., and containing 25 mM-citrate, pH 6. In this way, the final concentration of sodium butyl sulphate in the colorimetric-reaction mixtures was kept below 0·05 M. When SDS was tested in this way by mixing with the dialysed extract immediately before estimating phenolase a plot of the phenolase activity against wetting-agent concentration gave a curve closely resembling that obtained when the wetting agent was present in the colorimetric-reaction mixtures, but its position was shifted to the right so that the maximum activity was reached at a wetting-agent concentration some four times higher. It will be shown later (Table 2) that this effect is probably due to the fact that the ratio of the volume of extract to that of wetting agent is much higher when these are mixed before testing than when the wetting agent is present in the colorimetric-reaction mixtures. Since 0·1 M-sodium butyl sulphate does not significantly inhibit the phenolase activity and tests with this concentration in the colorimetric reaction mixtures failed to activate, it must be concluded that the activation curve of sodium butyl sulphate comparable with those of the other alkyl sulphates lies above 0·1 M but at a somewhat lower region than that shown in Fig. 3.

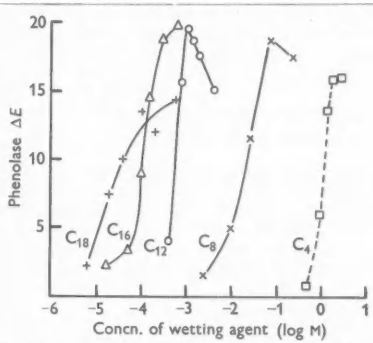

Fig. 3. Effect of sodium *n*-alkyl sulphates on the phenolase activity of dialysed water extract of broad-bean leaf at pH 6. +, Octadecyl; △, cetyl; ○, dodecyl; ×, octyl; activity measured colorimetrically with 0·011 ml. of extract under the conditions of Fig. 2. □, Butyl, activity measured as described in the text. DOPA as substrate. Temp., 18–19·5°.

The results suggest that over the range of chain length of 4–18 carbon atoms the concentration of sodium *n*-alkyl sulphate required to produce a given amount of activation of the latent phenolase decreases with increase in chain length. With the largest amount of sodium cetyl sulphate and the larger amount of sodium octadecyl sulphate all of the wetting agent was not in solution. The low solubility of sodium octadecyl sulphate is probably responsible for the anomalous shape of its activation curve.

Effect of time of exposure of the dialysed extract to wetting agent. Whereas the release of active phenolase from the dialysed extracts by the anionic wetting agents is a rapid process at relatively high concentrations of wetting agent, as the concentration decreases the rate of liberation of phenolase decreases.

At room temperature, 0·5 ml. of dialysed water extract of broad-bean leaf was added to 4·5 ml. of 15 mM-citrate containing varying amounts of SDS

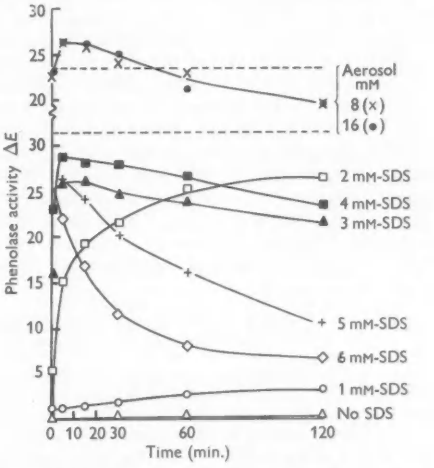

Fig. 4. Effect of time of exposure of dialysed water extract of broad-bean leaf to sodium dodecyl sulphate and Aerosol OT at pH 6 on the phenolase activity. Mixtures containing dialysed extract and wetting agent were maintained at 19–20·5° and the phenolase activity was estimated colorimetrically with DOPA at 19–20·5° at various times. ×, ●, Aerosol OT. △, ○, ◇, +, □, ▲, ■, sodium dodecyl sulphate. The horizontal broken lines show the activities obtained when the dialysed extract was added directly to the colorimetric reaction mixtures containing the wetting agent at 1·5 mM.

or Aerosol OT, pH 6. Immediately after mixing and again at later times, 0·15 ml. portions were taken and the phenolase activity was estimated colorimetrically with DOPA as substrate as described previously. A typical set of results is shown in Fig. 4. They show that the rate of release of active phenolase depends on the concentration of SDS. In the absence of SDS the increase in phenolase activity over a 3 hr. period is barely measurable. With increase in SDS there is an increase in the rate of release of active phenolase. In the experiment of Fig. 4 at a concentration of 4 mM-SDS or greater it is practically complete within 1 min. of mixing. The released phenolase is, however, unstable in these relatively high concentrations of SDS. This can be seen from the rapid loss of the released phenolase activity which takes place in the mixtures containing 5 and 6 mM-SDS. With Aerosol OT the results were similar to those obtained with SDS with the exception that high concentrations of Aerosol did not bring about a rapid loss of the liberated activity. The highest activities obtained by mixing the dialysed extract with SDS before testing were generally a little less than when the dialysed extract was added directly to colorimetric reaction mixtures containing SDS. The reverse was generally found with Aerosol OT (e.g. Fig. 4). The same pattern of response of the dialysed extracts to SDS and Aerosol OT was seen in different experiments but the response to a given concentration of agent varied slightly. This was most likely due to variation in the amounts and composition of the dialysed extracts used. In these experiments, the concentration of wetting agent which gave the maximum phenolase activity was higher than that required when the wetting agent was added directly to the colorimetric reaction mixtures. This may be because under the former conditions the volume of extract relative to that of the agent is much greater. It is shown in Table 2 that increasing the volume of dialysed extract diminishes the effect of both SDS and Aerosol OT.

The results of these experiments suggested that the negative results with the cationic and non-ionic wetting agents which were obtained in the previous experiments could have arisen if these agents are capable of liberating phenolase only at a slow rate. Accordingly CTAB and Triton X 100 were again tested. Mixtures containing CTAB at 0·5, 3 or 20 mM or Triton X 100 at 0·2, 1·2 and 8 g./l. final concentration and 0·5 ml. of dialysed water extract in a total volume of 5 ml., containing 20 mM-citrate, pH 6, were made up at room temperature and tested colorimetrically for phenolase activity. They were tested again after standing at room temperature for 10 min., 1 hr. and 3 hr. No measurable phenolase activity was found with any of these mixtures. In control mixtures with Aerosol OT at the same concentration as CTAB and made at the same time, large amounts of phenolase activity were found.

Effect of wetting agents on activated broad-bean phenolase. The failure to demonstrate activation of the latent phenolase in the dialysed water extracts by treatment with the cationic and non-ionic wetting agents could be explained if these agents inhibit or destroy active phenolase. Accordingly, the effect of these agents on an activated broad-bean phenolase preparation has been tested. It has been shown previously (Kenten, 1957) that when the precipitate obtained by saturating a water extract of broad-bean leaves with $(NH_4)_2SO_4$ is suspended in water and stored at 0°, the latent phenolase which it contains becomes active. For the tests described below a preparation corresponding to the second $(NH_4)_2SO_4$ precipitate of Kenten (1957) was aged at 0° in the presence of toluene for 6 weeks.

Table 2. *Effect of varying the volume of dialysed water extract of broad-bean leaf on the activation of phenolase by sodium dodecyl sulphate and Aerosol OT*

Mixtures containing varying amounts of dialysed water extract of broad-bean leaf and 2 mM-wetting agent in a total volume of 5 ml. of 15 mM-citrate, pH 6, were made up and kept at room temperature (20–21° with SDS, 16–16·5° with Aerosol OT). Samples equivalent to 0·015 ml. of dialysed extract in the experiment with SDS and 0·025 ml. in that with Aerosol were withdrawn at various times and the phenolase activity was estimated colorimetrically at room temperature with DOPA at pH 6. Different batches of dialysed extract were used in the two experiments. The amount of wetting agent carried over into the colorimetric reaction mixtures in the samples was insufficient to bring about appreciable activation of dialysed extract.

	Phenolase activity					
	SDS			Aerosol OT		
Extract (ml.) ...	0·1	0·5	1·5	0·2	0·8	3·2
Time of exposure (min.)						
0·5	19·0	5·0	1·0	25·0	20·0	3·5
5	24·0	11·5	1·0	32·0	26·5	3·5
15	20·5	13·5	1·0	31·5	27·5	4·5
60	12·0	19·0	1·5	21·0	26·0	8·0

The colorimetric method of estimating phenolase activity was used, the conditions being similar to those of Fig. 2. The concentration of wetting agent in the reaction mixtures was either 3 mM or 1·3 g./l. Under these conditions the rate of oxidation of DOPA was inhibited by 55–60 % in the presence of saponin, about 10 % in the presence of SDS and less than 10 % in the presence of Aerosol OT, Lissapol N, CTAB or cetylpyridinium bromide. With the exception of saponin, it is therefore unlikely that the failure to obtain activation with the non-ionic and cationic wetting agents was due to inhibition of active phenolase.

Effect of pH *on the capacity of Aerosol OT and sodium dodecyl sulphate to activate the latent phenolase of dialysed water extract of broad-bean leaf.* It has been shown (Kenten, 1957) that much active phenolase is liberated from extracts of broad-bean leaf by brief exposure to mildly acid or alkaline conditions (pH 3·5 or 11·5). In phosphate–citrate or pyrophosphate buffers over the range pH 5·5–8·5 there is little or no increase in the phenolase activity of a dialysed water extract of broad-bean leaf at about 20° during 2–3 hr. Furthermore, under these conditions there appears to be little loss of latent phenolase. Hence the effect of pH on the capacity of the anionic wetting agents to activate the latent phenolase can be studied over the range pH 5·5–8·5 without appreciable activation taking place in the absence of wetting agent.

In the experiments with Aerosol OT, mixtures containing 6 mM-Aerosol OT and 0·8 ml. of dialysed water extract of broad-bean leaf in a total volume of 5 ml., containing 10 mM-pyrophosphate buffer, were made up at various pH values and kept at room temperature (18–19°). The phenolase activity of 0·1 ml. samples of the mixtures was estimated colorimetrically with DOPA as substrate at different times. After 2 hr. latent phenolase plus active phenolase was estimated by taking 0·1 ml. samples into 4·9 ml. of 30 mM-citrate containing 5 mM-Aerosol OT, pH 6. Time (5 min.) was allowed for activation of the latent phenolase and then 1 ml. of DOPA (3 mg.) was added and the rate at which it was oxidized was followed colorimetrically and taken as a measure of latent plus active phenolase.

With sodium dodecyl sulphate a different batch of dialysed extract was used. At the pyrophosphate and wetting-agent concentrations used in the experiments with Aerosol OT a small crystalline precipitate formed in the mixtures at about pH 8. If the pyrophosphate concentration was decreased to 4 mM and the sodium dodecyl sulphate to 3 mM no precipitate formed at about pH 8, and these conditions were used in the experiments. In a total volume of 5 ml., 1 ml. of dialysed water extract was present and 0·15 ml. samples were withdrawn for phenolase estimations. At the end of the experiment, latent plus active phenolase was estimated in the same way as in the Aerosol OT experiments except that the sodium dodecyl sulphate concentration was 1·7 mM.

The results (Table 3) show that as the pH increases from 5·9 to 8·35 the rate of activation of the latent phenolase by the wetting agent decreases. The effect was more marked with Aerosol OT than with sodium dodecyl sulphate. Under the conditions used at about pH 8 the amount of activation by Aerosol OT is barely measurable in a 2 hr. period, whereas at pH 6 activation is virtually complete in 10 min. Such results could be obtained if the latent phenolase itself or the active phenolase derived from it were unstable at about pH 8. These possibilities are, however, excluded by the results of the estimations of latent plus active phenolase at the end of the experiments. These results show that after the 2 hr. incubation period

Table 3. *Effect of* pH *on the capacity of Aerosol OT and sodium dodecyl sulphate to activate the latent phenolase of dialysed water extract of broad-bean leaf*

Phenolase activity of mixtures containing dialysed water extract and Aerosol OT (6 mM) or SDS (3 mM) in pyrophosphate buffer was followed colorimetrically. Latent + active phenolase was estimated after the 2 hr. incubation period by treatment of samples of the mixtures with wetting agent at pH 6 before measuring their activity towards DOPA. Temperature 18–19°.

	Phenolase activity											
	With Aerosol				Without Aerosol				With SDS		Without SDS	
pH ...	5·9	6·8	7·65	8·35	5·9	6·8	7·65	8·35	5·9	8·25	5·9	8·25
Time of exposure (min.)												
0·5	22·5	2·0	0·5	0·5	0	0	0	0	24·0	1·0	0	0·5
10	30·0	2·0	1·0	1·0	—	—	—	—	26·0	2·0	—	—
30	31·5	5·0	0·5	1·0	—	—	0	0	24·5	2·5	—	—
60	30·0	6·0	1·0	1·0	0	0	0	0	22·5	3·5	—	0
120	29·5	8·0	1·5	1·5	0	0	0	0	16·5	4·5	0	0
Latent + active phenolase at 120 min.	33·5	30·5	29·5	29·5	31·0	30·0	29·5	28·0	12·0	22·0	19·0	22·0

almost as much phenolase can be liberated from the alkaline mixtures by treatment with wetting agent at pH 6 as that initially liberated in the mixtures at pH 6.

DISCUSSION

The results of the present work show that the latent phenolase of dialysed water extracts of broad-bean leaves can be activated by treatment with certain anionic wetting agents at pH 6. With the homologous series of sodium *n*-alkyl sulphates tested the concentration at which activation took place decreased with increase in chain length. Two properties of this series of compounds which appear to be especially relevant to the activation phenomenon are their capacity to form micelles and to combine with proteins. The critical concentration at which transition from simple strong electrolytes to micelles takes place with the sodium *n*-alkyl sulphates has been shown to decrease with increase in chain length from 12 to 18 carbon atoms (Powney & Addison, 1937). The critical concentration of micellar formation for SDS at about 20° is in the neighbourhood of 6 mM. With increase in salt concentration there is a steady decrease in the critical concentration, which at about 0·1 M-Na^+ ion concentration reaches a value of about 1·5 mM (Powney & Addison, 1937; Corrin & Harkins, 1947). The Na^+ ion concentration was less than 0·1 M in the experiments with SDS and in some activation of the latent phenolase was half maximal at 0·8 mM, and measurable activation was found at 0·2 mM, a concentration well below the critical point. Furthermore, the critical concentration of micellar formation for SDS is unaffected by variation in pH over the range 4·5–12 (Powney & Addison, 1937), whereas a change in pH from 6 to 8 greatly reduces the activation of latent phenolase by SDS. Therefore, although it is conceivable that the pronounced solvation properties of micelles could be connected with the activation of the latent phenolase, the results appear to exclude such a mechanism.

Kenten (1957) has discussed the reasons for believing that the known properties of the latent phenolase of extracts of broad-bean leaf are most readily explained by assuming that in the extracts there is either a phenolase–protein-inhibitor complex of the type described by Swartz, Kaplan & Frech (1956), in which the components are bound through interaction of ionic groups, or a phenolase precursor (prophenolase). It is generally thought that the ionic wetting agents produce their effects on proteins by combining with them primarily through electrostatic forces. With the anionic wetting agents there is much evidence that combination takes place at the cationic sites on the protein molecule with accompanying alteration of the net electrical charge (Klotz, 1953; Putnam, 1948). Therefore a phenolase–inhibitor complex held together by salt linkages might be expected to dissociate after combination with an anionic wetting agent. With a prophenolase such combination might permit configurational changes in the molecule which unmask the catalytic centre. In this connexion, it is of interest that the results of studies of the combination of albumin with large anions (Karusch, 1952) or SDS (Neurath & Putnam, 1945) suggest that unfolding or expansion of the protein molecule takes place as the number of bound ions increases.

The experimental results are in agreement with the suggestion that the anionic wetting agents activate through their capacity to combine with proteins. In the first place, no activation could be demonstrated with the non-ionic wetting agents tested. Secondly, it is known that the affinity of anions for proteins increases with the size of the anion (Steinhardt, Fugitt & Harris, 1942; Boyer, Ballou & Luck, 1947). This has been demonstrated with a variety of proteins and anions and in particular with octyl, decyl and dodecyl sulphates and serum albumin (Karusch & Sonenberg, 1949). Thus it would be expected that with increase in chain length the concentration of alkyl sulphate required to activate the latent phenolase would decrease.

It might be expected that a shift in pH from 6 to 8 would decrease the activation brought about by the anionic wetting agents since the net negative charge on a protein increases with increase in pH value. However, systems that have been tested experimentally do not all conform with this expectation. For example, although Boyer *et al.* (1947) found that the amount of sodium caprylate bound by albumin decreased with increase in pH from 6·8 to 11·8, Klotz, Burkhard & Urquhart (1952) found no pronounced change in the amount of methyl orange or azosulphathiazole anions bound by albumin over the range pH 6–9. If in fact activation of the latent phenolase does depend on combination of the anions at the cationic groups of proteins then the fact that a shift in pH value from 6 to 8 brings about such a large decrease in the amount of activation suggests that the iminazolium groups (pK 5·6–7·0; Cohn & Edsall, 1943) and terminal α-ammonium groups (pK 7·6–8·4; Cohn & Edsall, 1943) may be the sites at which combination takes place.

There is also diverse behaviour of protein towards the cationic wetting agents. These, through their capacity to combine at the negative sites on protein (e.g. Klotz, 1953), also might be expected to activate the latent phenolase. No evidence that they brought about any activation was obtained. Although Steinhardt & Zaiser (1950) have shown

that both cations and anions of about equal size are bound by wool, the failure of the cationic agents to activate latent phenolase would be consistent with the observation of Klotz, Gelewitz & Urquhart (1952) that organic cations of a wide variety of structures do not form complexes with serum albumin with an affinity even approaching that of complexes with anions of similar structure. Thus while the present results support the suggestion that the anionic wetting agents activate the latent phenolase by their ability to combine with proteins they do not help to decide whether a phenolase–inhibitor complex or a prophenolase is involved.

The latent phenolase of diapause grasshopper eggs is similar to that of extracts of broad-bean leaves in that activation follows treatment of it with anionic wetting agents such as Aerosol OT, sodium oleate and the long-chain alkyl sulphates (Bodine & Allen, 1938; Allen, Otis & Bodine, 1943; Bodine, Tahmisian & Hill, 1944; Bodine & Hill, 1945). The nature of the latent phenolase of grasshopper eggs has not yet been elucidated, but its properties are also consistent with the presence of either a phenolase–inhibitor complex or a prophenolase.

In the present work the maximum phenolase activities released by treatment of the bean-leaf extracts with Aerosol OT and sodium cetyl, dodecyl or octyl sulphates were about the same. It is not known whether these treatments do in fact bring about the activation of all of the latent phenolase present in the extracts. However, the results of preliminary tests suggest that treatment of the extracts with these anionic wetting agents leads to the release of about the same or a greater amount of phenolase than does activation by exposure to acid or alkaline pH or proteolytic enzymes (Kenten, 1957). This suggests that treatment of the extracts with the anionic wetting agents gives the best measure of latent phenolase. Furthermore, Aerosol OT is the preferred activating agent since it does not show the toxic effects at relatively high concentration found with alkyl sulphates.

SUMMARY

1. The latent phenolase of extracts of broad-bean leaf is activated by treatment with anionic wetting agents at pH 6. A number of cationic and non-ionic wetting agents were without effect.

2. With the homologous series of sodium *n*-alkyl sulphates tested, the concentration at which activation of the latent phenolase took place decreased with increase in chain length.

3. At pH 8 the rate of activation with sodium dodecyl sulphate and Aerosol OT was very slow or negligible compared with that at pH 6.

4. It is suggested that the anionic wetting agents activate through their capacity to combine with the cationic groups of protein. Dissociation of a phenolase–protein-inhibitor complex or configurational changes in a prophenolase might follow such combination.

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Formation of Specific Antibodies and γ-Globulin *in vitro*. A Study of the Synthetic Ability of Various Tissues from Rabbits Immunized by Different Methods

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Although there is abundant evidence that specific antibodies are formed in lymphoid tissues (e.g. spleen and lymph nodes) of immunized animals, relatively little is known about the extent to which different tissues contribute to overall synthesis of specific antibody or of other γ-globulin in the whole animal.

[^{14}C]Glycine has been shown to be incorporated into antibody by slices of spleen and liver taken from animals immunized intravenously (Ranney & London, 1951; Keston & Dreyfus, 1951), and by granulomatous tissue developed at the site of administration of antigen in an adjuvant mixture (Askonas & Humphrey, 1955). When the relative rates of incorporation of labelled amino acid into antibody by tissue slices taken from different organs of the guinea pig were compared with the populations of antibody-containing cells in those organs, Askonas & White (1956) found a generally good correlation between the two measurements. There is therefore some justification for considering that the ability of tissues to incorporate amino acids into specific antibody or other γ-globulin *in vitro*, under suitable experimental conditions, is a measure of the relative ability of the tissues to synthesize these proteins. Because of its simplicity we have used this method to study antibody synthesis by different tissues in rabbits immunized with one or more antigens and by different routes. Our results show that the pattern of antibody synthesis varies widely under different conditions, but that it is always accompanied by increased synthesis of other γ-globulin. The reasons for this and the mechanism of formation of these proteins by the tissue slices are discussed.

METHODS

Immunization of animals

Rabbits (albino or sandylop strains) were bred at the National Institute for Medical Research and were fed on pelleted Diet no. 18 (Bruce & Parkes, 1940). They weighed 2·0–2·8 kg. at the time of use.

Intravenous immunization. Pneumococci type 3 killed with formalin (Kauffmann, Björneboe & Vammen, 1938) were injected intravenously as a suspension containing 4×10^9 organisms/ml. Increasing amounts (0·1–1 ml.) were given thrice weekly for 3–4 weeks. In some rabbits the courses of injection were repeated after a rest period of 2 months.

Ovalbumin [four times recrystallized, and precipitated with alum (Porter, 1955)] was administered according to a schedule similar to that used for pneumococci. The amounts injected on each occasion were increased gradually from 0·5 to 5 mg.

Immunization with Freund's adjuvant. Ovalbumin was emulsified in a modification of Freund's adjuvant mixture (Freund & McDermott, 1942) by using Unguentum Alcohol Lanis B.P. and Light Liquid Paraffin B.P. The final concentration of ovalbumin was 10 mg./ml. and of heat-killed tubercle bacilli 1 mg./ml. Two injections of 1 ml. were given either intramuscularly or intraperitoneally at intervals of 1 week. The precipitating antibody content of the serum rose gradually for 6–8 weeks after the last injection, and then remained fairly constant for several weeks. Large cystic granulomatous masses (weighing 30–100 g.) developed at the injection sites.

A few animals received one or two intravenous injections of alum-precipitated ovalbumin 4 weeks after intramuscular administration of ovalbumin in adjuvant.

Serum-antibody levels were measured by quantitative precipitation in the presence of slight excess of antigen. After standing overnight at 2° the precipitate was washed three times at 0° and dissolved in 0·1 N-NaOH, and the amount of protein was estimated by measuring the absorption at 280 mμ.

Tissue-slice technique

^{14}C-*Labelled amino acids.* Biosynthetic [^{14}C]glycine and L-[^{14}C]valine were obtained from the Radiochemical Centre, Amersham, Bucks, with specific activities of 56 and 53 μc/mg. respectively.

Preparation of tissue slices. The immunized rabbits were bled from the ear exhaustively and then killed by injecting 120 mg. of pentobarbital intravenously. The organs to be tested were removed immediately, rinsed in cold 0·9% NaCl solution and chilled on ice. After each tissue had been weighed, appropriate amounts were chopped finely (to about 0·3 mm.) with an automatic chopper (McIlwain & Buddle, 1953) and with small scissors by hand. Portions of chopped tissue (0·3–1 g.) were weighed and incubated as described below.

Incubation of tissues. The tissues were incubated in Hanks's medium (Hanks, 1948), containing 0·3 g. of glucose, a complete amino acid mixture (5 mg. of N), prepared from acid-hydrolysed rabbit plasma protein with

added tryptophan, and 5000 units of penicillin in 100 ml. Each portion of tissue was suspended in 0·5 ml. of the animal's own serum and 4·5 ml. of medium containing 2–3 μc of [^{14}C]glycine or L-[^{14}C]valine in silicone-treated flasks. The flasks were gently shaken at 37°, and gassed continuously with $O_2 + CO_2$ (95:5, v/v) mixture. At the end of the incubation Merthiolate (Eli Lilly and Co.) was added to a concentration of 10^{-4} M, together with several milligrams of inactive amino acid to dilute ^{14}C-labelled amino acid in the medium and so to diminish adsorption of the label on protein precipitates later. In order to break up cells the incubation mixture was frozen in an ethanol–solid CO_2 mixture at −70° and then allowed to thaw. Carrier antibody was provided for each flask by addition of antiserum or purified γ-globulin containing antibody (sufficient to yield a total of 5–10 mg. of antigen–antibody precipitate). The contents of each flask were homogenized for 1 min. in a Potter-type homogenizer, and the total volumes adjusted to 10–12 ml. with M/15 phosphate buffer, pH 7·2. Sediment was removed by centrifuging for 20 min. at 18 000 *g* at 3°, and the supernatant was freed of fat by centrifuging for 20 min. at room temperature at 1500 *g*; the clear extract was used for isolation of antibody and other γ-globulin. In certain cases the sediment was treated as described below under 'Preparation of tissue residue'.

Isolation of specific antibody and other γ-globulin

Precipitable specific antibody was isolated by addition of a slight excess of antigen (one-tenth of the weight of antibody for ovalbumin and one-twentieth for pneumococcal capsular polysaccharide), followed by incubation of the tubes for 30 min. at 37° and 18 hr. at 1°. The specific precipitates were centrifuged, washed three times with 0·9% NaCl solution containing inactive amino acids, then with 50% aqueous ethanol, with hot ethanol and finally with ether. They were dried at room temperature.

The remaining γ-globulin was precipitated with 18% (w/v) Na_2SO_4; after being redissolved in a smaller volume it was reprecipitated with 16% and finally with 12% (w/v) Na_2SO_4, according to the method described for human γ-globulin by Kekwick (1940). The product was dialysed against distilled water, dried from the frozen state, and redissolved in 0·9% NaCl solution. Insoluble material was removed by centrifuging and to the supernatant was added 10 vol. of ethanol at 0°. The precipitated protein was washed with 50% ethanol, ethanol and ether and dried. The freeze-dried material was analysed electrophoretically in veronal buffer, pH 8·6, *I* 0·1. It corresponded to the components of rabbit serum moving slowest during electrophoresis at pH 8·6, and was not evidently contaminated with any other globulins or with albumin.

Preparation of tissue residue

The insoluble material, which was sedimented after homogenization, was washed with 0·9% NaCl solution, and protein was then precipitated by addition of trichloroacetic acid to a concentration of 5% (w/v). The precipitate was washed twice with boiling trichloroacetic acid solution, followed by hot and cold ethanol, and finally dried with ether.

Determination of radioactivity

Dried protein was plated at infinite thickness on 0·3 or 1 cm.^{2} disks, and the radioactivity determined by counting on an automatic recording Geiger counter with thin end-window, to an accuracy of at least ±5%. When necessary, counts were corrected to infinite thickness.

Calculation of relative capacity of tissues to form antibody

The specific radioactivity of the isolated antibody was found to be proportional to the amount of tissue incubated (as was to be expected, since the weight of newly formed radioactive antibody was negligible compared with the weight of inactive carrier-antibody added). Hence the relative synthetic abilities of the tissues could be compared by calculating the radioactivity of the isolated antibody/unit wt. (0·1 g.) of tissue incubated. As a standard of comparison for all the experiments, spleen was chosen arbitrarily to represent 100% activity, and the percentage activity of other tissues was determined relative to it.

In one experiment tissue slices were incubated separately with [^{14}C]glycine and L-[^{14}C]valine, and the relative synthetic activities of the tissues were compared, on the basis of their abilities to incorporate either of these amino acids into antibody. Although the absolute levels of radioactivity were not the same for each amino acid, the relative synthetic abilities were the same within experimental error.

RESULTS

Time-course of incorporation of [^{14}C]*amino acids into protein*

Fig. 1 shows the course of incorporation of [^{14}C]-amino acid into specific antibody, other γ-globulin and tissue-debris fractions when slices of lymph glands from an animal immunized against ovalbumin were incubated for varying periods of time in the presence of ^{14}C-labelled amino acids. It may be seen that the specific radioactivities of the antibody and γ-globulin follow an S-shaped curve. During a preliminary period of 10–15 min. no radioactivity appears, whereas after 30 min. radioactivity increases steeply for 2 hr., after which the rate of increase falls off. Incorporation of radioactivity into insoluble tissue protein, however, follows a straight line throughout the period of the experiment. This suggests that the slices lose the capacity to form antibody and γ-globulin earlier than the capacity to incorporate [^{14}C]amino acids into insoluble particles and structural components. The length of time during which incorporation of [^{14}C]amino acids into antibody protein continued varied with different tissues; chopped granuloma tissue (from the site of injection of the adjuvant mixture) continued to incorporate [^{14}C]glycine into antibody for up to 6 hr. and spleen slices for more than 4 hr. (see Fig. 2). In experiments designed to compare the antibody-forming capacity of different tissues an incubation time of 3 hr. was therefore chosen.

It must be emphasized that the absolute values for specific radioactivities depend greatly upon the

amount of carrier protein added. Since there was added two to three times more 'normal' γ-globulin than antiovalbumin, the absolute rates of incorporation into these two proteins in Fig. 1 were in fact quite similar.

The tissue protein not extracted with saline represents a complex mixture of structural proteins and proteins associated with subcellular particles. No carrier protein was added to this fraction, hence the specific antibody and γ-globulin secreted by the cell had a much higher specific radioactivity than the tissue protein.

Effects of cell damage on incorporation

Rapid freezing and thawing of the tissues before slicing reduced the uptake of radioactivity into antibody on subsequent incubation by 90–95%.

On several occasions the effect was examined of homogenizing spleen, bone marrow and lymph glands for 15–30 sec. in isotonic salt media, or in 0·25 or 0·29 M-sucrose, or in the sucrose medium containing inorganic salts described by Zamecnik & Keller (1954). After nuclei and whole cells had been centrifuged at 1500 *g* for 10 min. the supernatant containing mitochondria and microsomes was incubated aerobically, or anaerobically in the medium of Zamecnik & Keller (1954) with 3 μc of added [^{14}C]glycine or L-[^{14}C] valine. In no case was incorporation of ^{14}C into specific antibody or other γ-globulin observed.

Even slight damage to cells appeared greatly to diminish uptake of radioactivity into antibody. For example, when a suspension of cells was obtained by teasing out cells from a lymph gland by means of a needle, the cells looked somewhat damaged under the microscope; upon subsequent incubation under standard conditions very little ^{14}C was incorporated into antiovalbumin or into other γ-globulin, whereas considerable uptake took place into insoluble tissue protein (Table 1). This was in contrast with experiments with sliced tissues, and suggests either that antibody-producing cells were not teased out from the gland or, more probably, that under our conditions whole undamaged cells are necessary for formation of extractable antibodies and other γ-globulin.

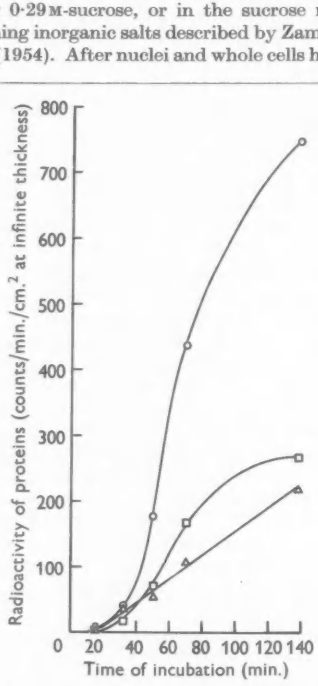

Fig. 1. Time-course of incorporation of [^{14}C]glycine into antiovalbumin, γ-globulin and insoluble tissue protein by sliced lymph glands. Portions (0·35 g.) of sliced lymph glands (from a guinea pig immunized with ovalbumin in adjuvant) were incubated in the usual medium (see Methods) containing 2·5 μc of [^{14}C]glycine. ○, Antiovalbumin; □, other γ-globulin fraction; △, tissue protein not extractable by isotonic saline–phosphate.

Specificity of incorporation of [^{14}C]*amino acids into antibody*

To test the extent of contamination of the antigen–antibody precipitates obtained by the technique described, antibody against the capsular polysaccharide of pneumococci type 3 was added to tissue slices from animals immunized against ovalbumin; after incubation for 3 hr. in the usual way, each antibody was isolated by precipitation with the appropriate antigen. The pneumococcal antibody was precipitated first, and had less than one-tenth of the radioactivity of the antiovalbumin.

Table 1. *Effect of damage to cells on incorporation of* [^{14}C]*amino acids into antibody and γ-globulin*

Lymph glands (pooled) from a rabbit immunized subcutaneously with ovalbumin in adjuvant. Incubations were carried out in the presence of 2·5 μc of [^{14}C]glycine in the usual medium except where otherwise indicated.

	Radioactivity, counts/min./cm.² (at infinite thickness)		
Lymph gland	Anti-ovalbumin	γ-Globulin	Residual tissue protein
Slices (0·35 g.)	762	320	220
Damaged cells*	8	25	435
Homogenate†	0	2	53

* Cells were teased from lymph glands (1 g.) by means of a needle.

† Homogenate was prepared from lymph glands (1 g.) and incubated in 0·29 M-sucrose–salt medium (Zamecnik & Keller, 1954). Nuclei and whole cells were removed by centrifuging at 500 *g* for 10 min. at 1°.

This finding is similar to that of Keston & Dreyfus (1951) and Ranney & London (1951), and even the small amount of radioactivity found in the pneumococcal antibody was presumably due to coprecipitation of some labelled γ-globulin. No exchange occurred of amino acids in the medium with protein added to the incubation mixture. When ^{14}C-labelled antibody to pneumococci polysaccharide was added to granuloma slices from an animal immunized against ovalbumin, and reisolated after incubating for 1 min., and 6 hr. in the absence of added [^{14}C]amino acids, the radioactivities of the antibody protein were 1870 and 1880 counts/min./cm.^{2} respectively.

Secretion of antibody into incubation medium by slices

In the experiments discussed above, antibody isolated at the end of an experiment included antibody secreted into the medium as well as intracellular antibody. In order to study secretion and total formation of labelled antibody separately, slices of spleen were incubated with [^{14}C]glycine and after various time intervals the cellular components were sedimented by centrifuging for 15 min. at 15 000 *g*. Equal amounts of a γ-globulin concentrate containing antiovalbumin were added to the supernatant and to the tissue sediment (washed once with the medium). Antibody was isolated directly from the supernatant in the usual way, and from the sediment after further addition of 3–4 ml. of buffer solution, followed by freezing and thawing and homogenization to break up the cells. The radioactivities of the extracellular and intracellular antibody, as isolated, are shown in Fig. 2. Since in each case the antibody formed by the tissue slices was diluted with a very much larger amount (6 mg.) of added antibody, the observed radioactivities are not the actual radioactivities of the antibody formed but give an indication of the amounts of [^{14}C]glycine incorporated. After 30 min. radioactivity was present in the intracellular antibody, but no radioactive protein had been secreted into the medium. After 3 hr. the radioactivity of the intracellular antibody increased only slightly, whereas that of the secreted antibody continued to rise at a constant rate for 4 hr. These findings are compatible with the idea that the total pool of intracellular antibody remained constant, while the specific radioactivity gradually rose by formation of new molecules with the same specific activity as that of the intracellular free amino acids. The radioactivity of the extracellular antibody represents the accumulation of all the antibody secreted, with gradually increasing specific activity. Between the third and the fourth hours, when the radioactivity of intracellular antibody remained fairly constant, the accumulation of radioactivity in the secreted antibody was equal to about half the radioactivity of the antibody in the cells. If the specific activity of secreted and intracellular antibody were equal during this period (which would be approximately true on the assumptions stated above) this would mean that an amount of antibody equal to the intracellular pool would be secreted in about 2 hr. This value is in good agreement with the time of about 2 hr. deduced for secretion rates *in vivo* by Humphrey & Sulitzeanu (1957).

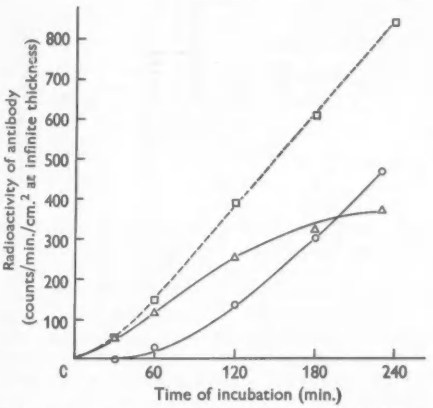

Fig. 2. Incorporation of [^{14}C]glycine into intracellular and secreted antiovalbumin by spleen slices. ○, Secreted antibody isolated from medium; △, intracellular antibody extracted from spleen slices after homogenization; □, combined intracellular and extracellular antibody. Slices of spleen (0·5 g.) from a rabbit immunized intravenously were incubated in the usual medium containing 3 μc of [^{14}C]glycine. Antiovalbumin carrier (6 mg.) was added before isolation of each antibody fraction.

The general pattern of secretion of radioactive antibody agrees with the results obtained by Miller, Bly, Watson & Bale (1951), who studied incorporation of [^{14}C]amino acids into albumin by isolated perfused rat liver, and with our own findings on antibody formation by perfused rabbit lung (B. A. Askonas & J. H. Humphrey, unpublished work). This suggests that, for a while at least, slices behave similarly to whole organs during perfusion.

Relative ability of various tissues to form antibody and other γ-globulin after immunization by different methods

Tissue slices were incubated with ^{14}C-labelled glycine or valine for 3 hr. periods and the antibody and other γ-globulin then isolated. Within each experiment the results on the various tissues are comparable, but because the amount of

intracellular plus carrier antibody varied from animal to animal the results of one experiment cannot be directly compared with those of another. The relative synthetic abilities of tissues/unit weight were compared and the contributions by the organs tested to the overall synthesis of specific antibody and other γ-globulin in the whole animal could be assessed. Whereas the weights of spleen, lung, liver, kidney and individual lymph glands can be determined easily, those of the total bone marrow and total lymph glands cannot. For these last two tissues we have therefore assumed weights of 30 g. of bone marrow and 3 g. of lymph glands. These values are based on the estimates that total bone marrow is equal to 1·5–1·7% of the body weight (Nye, 1931–32; Dietz, 1944) and that total lymphoid tissue is equivalent to about 0·15% of body weight (Yoffey & Courtice, 1956); they are lower than the estimates quoted to allow for the probability that not all the bone marrow and lymphoid tissue was as active as the femoral marrow and the enlarged lymph glands examined.

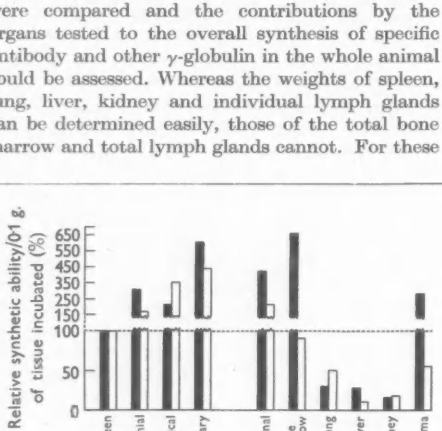

Fig. 3

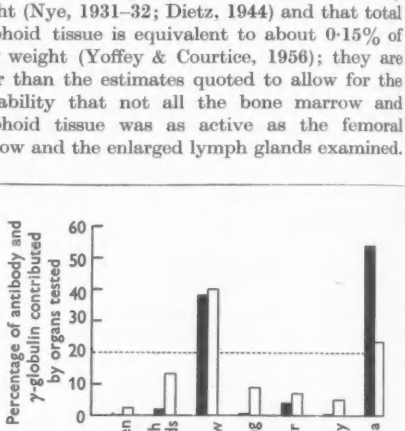

Fig. 4

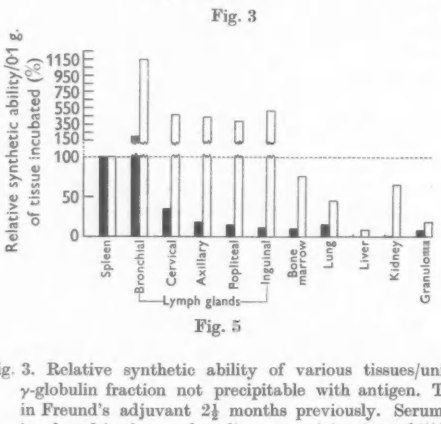

Fig. 5

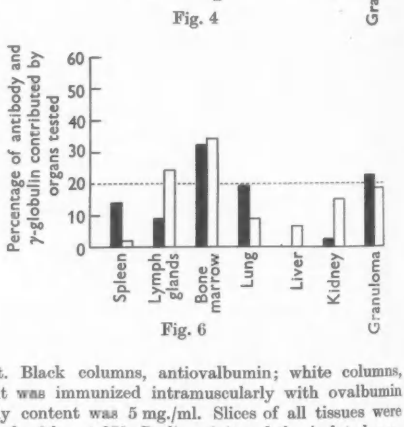

Fig. 6

Fig. 3. Relative synthetic ability of various tissues/unit weight. Black columns, antiovalbumin; white columns, γ-globulin fraction not precipitable with antigen. The rabbit was immunized intramuscularly with ovalbumin in Freund's adjuvant $2\frac{1}{2}$ months previously. Serum-antibody content was 5 mg./ml. Slices of all tissues were incubated in the usual medium, containing 2 μC of [^{14}C]glycine, for 3 hr. at 37°. Radioactivity of the isolated proteins after incubation of 0·1 g. of sliced spleen was chosen arbitrarily to represent 100% activity.

Fig. 4. Proportion of antibody and γ-globulin formed by each organ tested. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Rabbits were immunized intramuscularly with ovalbumin in Freund's adjuvant. The relative contribution made by each organ was calculated by considering the total weight of the organ in the rabbit and the relative synthetic abilities of the tissues (cf. Fig. 3).

Fig. 5. Relative synthetic ability of various tissues/unit weight. Ovalbumin was administered in Freund's adjuvant, and later intravenously. Black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit was injected twice with alum-precipitated ovalbumin intravenously 4 weeks after intramuscular administration of ovalbumin in Freund's adjuvant. The antiovalbumin content of the serum was 5·6 mg./ml. Conditions of incubation and expression of results were as in Fig. 3.

Fig. 6. Percentage of antibody and γ-globulin contributed by organs tested. Ovalbumin was administered in Freund's adjuvant and intravenously. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Relative synthetic abilities of the tissues/unit weight for the same animal are presented in Fig. 5.

It must be recognized that these are approximations, but any errors are unlikely to affect the overall picture very greatly. The results below are typical of those found in repeated experiments.

Ovalbumin in Freund's adjuvant. Rabbits which had been injected either intramuscularly or intraperitoneally 2–3 months previously with ovalbumin in adjuvant were examined. Before the granulomatous tissue at the injection site was tested, it was freed from the thick viscous fluid containing necrotic cells which accumulates within it. The results of a typical experiment are shown in Figs. 3 and 4. In this rabbit, which had 5 mg. of specifically precipitable antibody/ml. of serum, the antibody-forming activity/unit weight of tissue in the spleen was low, but nevertheless was three to six times that of lung, liver or kidney. Granuloma tissue, however, was three times, lymph glands two to six times and bone marrow six times as active as spleen. The production of other γ-globulin followed a similar pattern to that of specific antibody, with the exceptions that granuloma and bone marrow were less active/unit weight than spleen. On the basis of their total weight in the whole animal, the granuloma tissue contributed a major part of the specific antibody, and bone marrow most of the other γ-globulin.

Ovalbumin in Freund's adjuvant plus intravenous alum-precipitated ovalbumin. It was observed that if animals previously injected intramuscularly with ovalbumin in adjuvant were given a short intravenous course of alum-precipitated ovalbumin, the antibody content of the serum rose rapidly to a level some five times as high. Since this suggested that the pattern of production by different tissues might have changed, this possibility was investigated. A rabbit which had received ovalbumin in adjuvant intramuscularly 4 and 5 weeks previously was given two intravenous injections of 1·5 mg. of alum-precipitated ovalbumin intravenously.

The serum content of precipitable antibody rose from 1·1 mg./ml. before the intravenous injections to 5·6 mg./ml. 1 week later. The findings in this animal are presented in Figs. 5 and 6. It will be seen that, in contrast with the previous experiment, the spleen, per unit weight, was now very much more active in forming antibody than any tissue examined except the bronchial lymph gland. As regards overall antibody production, granuloma tissue was now relatively less important, and both spleen and lung contribute significant amounts, in addition to bone marrow. The change in the pattern of overall production of other γ-globulin was much less.

Intravenous immunization with a single antigen. Figs. 7 and 8 illustrate the results obtained with tissues from a rabbit which had received nine intravenous injections of alum-precipitated ovalbumin (total 8 mg.) during the course of 3 weeks. The serum taken 2 days after the last injection contained 1·2 mg. of precipitable antibody/ml. In this animal the bone marrow formed most of the specific antibody and other γ-globulin. The apparent contribution of the liver may be spurious, since the specific radioactivities of the antibody isolated from liver slices was so low as to be of doubtful significance.

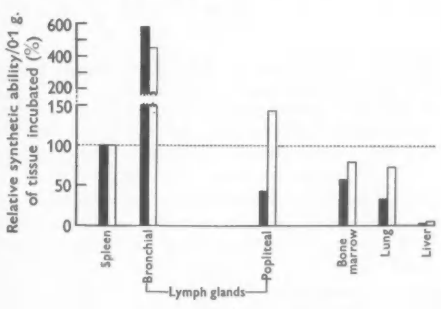

Fig. 7

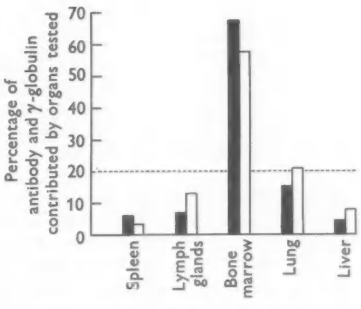

Fig. 8

Fig. 7. Relative synthetic ability/unit weight of various tissues from an intravenously immunized rabbit. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Nine intravenous injections of alum-precipitated ovalbumin were given over a period of 3 weeks; the serum contained 1·2 mg. of antiovalbumin/ml. Conditions of incubation and expression of results were as in Fig. 3, except that 3 μc of L-[^{14}C]valine was used for the incubation.

Fig. 8. Proportion of antibody and γ-globulin formed by organs tested, after intravenous immunization of a rabbit. Black columns, antiovalbumin; white columns, other γ-globulin fraction. For conditions of immunization with alum-precipitated ovalbumin and relative synthetic ability of the tissues/unit weight, see Fig. 7.

Intravenous immunization with two antigens. In order to compare the production of antibody by different tissues against two different antigens in the same animal, a rabbit was injected intravenously on alternate days with formalin-killed pneumococci type 3 or alum-precipitated ovalbumin. Four days after a 3 weeks' course of such injections the serum (1 ml.) contained 2 mg. of antibody to ovalbumin and 18 mg. of antibody to capsular polysaccharide. Tissue slices were incubated in the usual way, except that the added carrier contained known amounts of antibody to capsular polysaccharide, antiovalbumin and other γ-globulin. The two antibodies were isolated successively from the same incubation mixtures. The results of this experiment are shown in Figs. 9

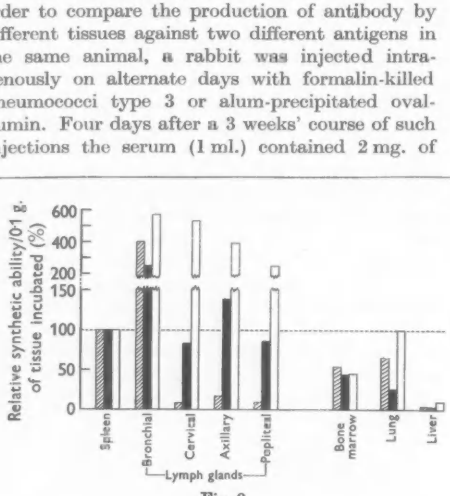

Fig. 9

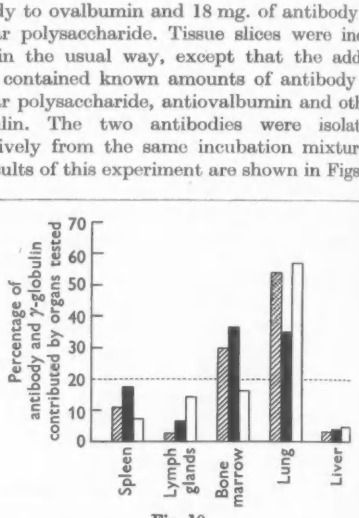

Fig. 10

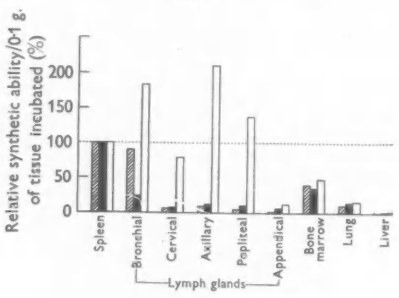

Fig. 11

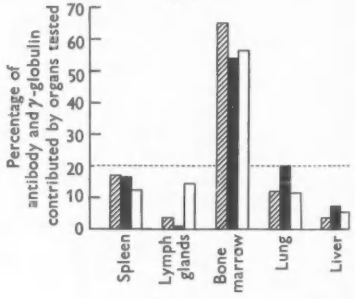

Fig. 12

Fig. 9. Relative synthetic ability of various tissues in a rabbit immunized intravenously with two antigens. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit received intravenous injections of formolized type 3 pneumococci and alum-precipitated ovalbumin on alternate days for 3–4 weeks. Antibody levels: 2 mg. of antiovalbumin and 18 mg. of antibody to capsular polysaccharide/ml. of serum. Conditions of incubation and expression of results were as in Fig. 3. L-[^{14}C]Valine (2 μc) was used for the incubation.

Fig. 10. Proportion of antibody and γ-globulin formed by different organs tested (intravenous alum-precipitated ovalbumin and type 3 pneumococci). Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. For conditions of immunization of the rabbit and relative synthetic ability of the tissues/unit weight see Fig. 9.

Fig. 11. Relative synthetic ability of various tissues in a rabbit immunized intravenously with two antigens. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit received intravenous injections of formalin-killed type 3 pneumococci and alum-precipitated ovalbumin as in Fig. 9, after a course of injections of pneumococci 9 months previously. Antibody levels: 4·3 mg. of antiovalbumin and 7·6 mg. of antibody to capsular polysaccharide/ml. of serum. Conditions of incubation and expression of results were as in Fig. 3.

Fig. 12. Proportions of antibody and γ-globulin formed by different organs tested after intravenous immunization with alum-precipitated ovalbumin and type 3 pneumococci. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. See Fig. 11 for conditions of immunization of the rabbit and relative synthetic ability of the tissues/unit weight.

and 10. The most interesting feature, as regards the relative synthetic activities/unit weight of tissue, is the marked variation between the different lymph glands examined, which must presumably reflect some differences in access of the two antigens to these glands. A striking aspect of Fig. 10 is the very large contribution made by the lung to the overall synthesis of antibody to capsular polysaccharide and of γ-globulin, both being more than half of the total. In this rabbit both the lung and spleen were hypertrophied to three to four times their normal weights, a finding which is not uncommon when rabbits are immunized intravenously with pneumococci, and which has been observed by Humphrey & Sulitzeanu (1958) in their study of rates of antibody synthesis *in vivo*.

In animals in which such hypertrophy did not occur the picture after intravenous immunization was somewhat different. Figs. 11 and 12 illustrate an experiment carried out with tissues from a rabbit which had received one course of injections of pneumococci 9 months before a course of alternating injections of pneumococci and alum-precipitated ovalbumin as described above. The antibody content of the serum at death was 7·6 mg. of antibody to capsular polysaccharide/ml. and 4·3 mg. of antiovalbumin/ml. In this animal the contribution of the lung to antibody and γ-globulin synthesis was relatively small. It is also noticeable that the relative synthetic abilities of the lymph glands examined were much more alike.

DISCUSSION

Significance of incorporation of [^{14}C]*amino acids into proteins in systems* in vitro

We have assumed that incorporation by tissue slices of ^{14}C-labelled amino acids into specifically isolated proteins represents a true synthesis of these proteins. There has been much discussion (see, for example, Tarver, 1954) of whether such an assumption is justified, or whether incorporation does not rather represent exchange of amino acids of the proteins without true synthesis. Our control experiments showed that tissues from rabbits immunized against ovalbumin, but not against pneumococci, incorporate [^{14}C]glycine into antiovalbumin, but not into pneumococcal antibody added to the reaction mixture. Thus exchange between extracellular protein and the amino acids of the medium can be ruled out. Radioactivity in the antibody was not due to non-specific protein co-precipitated with the antigen–antibody precipitate, since no radioactivity was found in antibody isolated after incubation of liver slices; yet liver slices are known to be active in protein synthesis. Furthermore, breaking up of the cells, or even slight damage, prevented incorporation into specific antibody and other γ-globulin, even when incorporation into insoluble tissue protein still occurred. For these reasons we consider that the incorporation of ^{14}C into specific soluble proteins which we observed was in fact a measure of true synthesis.

Since the slices were incubated for only 3 hr. no attempt was made to demonstrate net formation of antibody, such as was shown by Stavitsky (1955), and by Steiner & Anker (1956), using conditions under which lymph cells and spleen cells remained active for 1–3 days. However, it was possible to calculate the amount of antibody which might be formed, as the following example shows.

After incubation of spleen slices with 2·8 μc of [^{14}C]glycine for 3 hr., 5 mg. of antiovalbumin (including carrier antibody) was isolated, having a radioactivity equivalent to 7500 counts/min./cm.^{2} (at infinite thickness) for 1 g. of incubated tissue.

Since our counter gave 1000 counts/min. for a 1 cm.^{2} standard containing 10^{-3} μc/mg. (at infinite thickness), the isolated antibody had an activity of $7{\cdot}5 \times 10^{-3}$ μc/mg., i.e. 5 mg. of antibody contained $37{\cdot}5 \times 10^{-3}$ μc. The medium contained 2·8 μc of [^{14}C]glycine and a total of 120 μg. of glycine. Thus 1·3 % of the glycine (or 1·6 μg.) was incorporated into the antibody. Since rabbit antibody contains 5 % of glycine (Smith, McFadden, Stockell & Buettner-Janusch, 1955), about 32 μg. of antibody would have been newly formed by 1 g. of spleen. This is probably an underestimate, since it ignores dilution of added isotope by intracellular amino acids. Antibody produced by cells cultured for 2 days in medium containing [^{14}C]glycine was found by Steiner & Anker (1956) to have about half the isotope concentration, calculated on the assumption that all the glycine residues of the antibody were derived from the added labelled glycine. But even if the intracellular glycine from which the new antibody was formed had a lower specific activity than the glycine in the medium, only microgram quantities would have been newly formed by 0·5 g. of spleen, and so small an increase would be difficult to detect under our conditions of isolating the antibody by precipitation with antigen.

Mechanical damage to the cells stopped incorporation of [^{14}C]amino acids into specific antibody or other γ-globulin, while considerable uptake into the insoluble tissue protein persisted. When the cells were homogenized in various media with a Potter–Elvehjem-type homogenizer with a polytetrafluoroethylene pestle, no incorporation into these soluble proteins could be demonstrated, and only a much smaller incorporation

into the insoluble proteins. Whole cells therefore seem to be necessary for formation of specific soluble proteins, or at least for their release from subcellular particles. Other workers studying subcellular fractions of tissues have found similar results. Littlefield, Keller, Gross & Zamecnik (1955) found that during the first 20–30 min. the insoluble protein of liver microsomes became labelled *in vitro*, but that there was very little activity in the soluble proteins. Stephenson, Thimann & Zamecnik (1956) likewise found that the microsomal fraction of tobacco leaves infected with tobacco-mosaic virus incorporated radioactive amino acids into insoluble proteins but not into those of the virus. In contrast with our findings, Ogata, Ogata, Mochizuki & Nishigama (1956) report that cell-free homogenates of popliteal lymph glands (obtained by grinding with glass powder) incorporated [^{14}C]glycine into antibody to a greater extent and for a longer time than into tissue protein or nucleoprotein fractions. These results are at variance also with the findings of other workers who have studied synthesis of soluble proteins in broken-cell systems.

The exact significance of the shape of the curves obtained when incorporation of [^{14}C]amino acids into antibody is plotted against time (Figs. 1, 2) is difficult to assess. Since the antibody was isolated by addition of a very large excess of carrier the ordinates represent total incorporation. There is undoubtedly a lag period before the appearance of radioactive antibody and γ-globulin, lasting about 10–15 min. (Figs. 1, 2). How much of this is due to the time required for the cells to recover after being chilled and handled, and how much to preliminary formation of cell-bound compounds as postulated by Peters (1953), it is impossible to state.

Variation in sites of synthesis with different methods of immunization

In untreated rabbits, slices of various tissues (spleen, lymph glands, bone marrow) have been shown to incorporate ^{14}C-labelled amino acids into the γ-globulin fractions normally found in the serum, whereas slices of liver and kidney showed no such activity (Askonas, Humphrey & Porter, 1956). The same tissues that form the γ-globulin normally found in serum may be stimulated to form specific antibodies after administration of antigen, and in general we have found that stimulation of production of specific antibody was accompanied by an equal or even greater production of γ-globulin not precipitable with antigen. The question arises, how much of the other γ-globulin is in fact unprecipitated antibody against the specific antigen or impurities. In unpublished experiments we found that animals immunized as described formed negligible amounts of precipitable antibody against pneumococcal antigens other than the capsular polysaccharide, or against possible contaminants of ovalbumin such as conalbumin or ovomucoid. Using the technique of Farr (1956) we failed to detect significant non-precipitating antibodies against thrice recrystallized ovalbumin. There are no grounds therefore for stating that a substantial part of the other γ-globulin was specific antibody against the injected antigen, and its nature remains uncertain.

The major sites of antibody formation varied with the route of injection of the antigen. However, most active synthesis took place in the tissues which may be expected to take up the largest amount of antigen after injection by different pathways, provided that these tissues are capable of undergoing the type of cellular proliferation required for antibody formation. The liver is an exception: antigen accumulates in the Kupffer cells, but induces barely any antibody formation. A possible explanation is that antigen uptake by Kupffer cells is so efficient as to preclude its uptake by other types of cells, e.g. the primitive reticulum cells, described by Marshall & White (1950), which may be a necessary preliminary to antibody synthesis.

When ovalbumin was administered in Freund's adjuvant intramuscularly, the spleen and lung made only traces of antibody (Figs. 3, 4). After two intravenous injections of antigen, the spleen had a higher synthetic ability than the other tissues tested except for the bronchial lymph node (Figs. 5, 6). The relative contribution of the spleen and lung increased from a trace to 15 and 20% respectively. The responses shown by the spleen, lung and bronchial lymph node suggest that these tissues contained cells which had been 'conditioned' by trace amounts of antigen released from the adjuvant site but that proliferation of plasma-type cells and the production of large amounts of antibody by these tissues could take place only when more antigen was taken up by these tissues themselves (i.e. after intravenous injection).

Even when two particulate antigens, one in the form of killed bacteria and one adsorbed on to particles, are injected by the same route, intravenously, it is clear that their subsequent distribution must become different, since quite large variations were observed in the synthetic abilities of tissues for the two antibodies (Fig. 9). The lung is the main capillary filter bed in the body, and it is not surprising that the draining lymph nodes regularly showed such high activity. We are unable to generalize, however, about what conditions will cause the enormous proliferation of

antibody-forming cells in the lung itself, which was frequently, but not always, observed in animals given prolonged intravenous injections of pneumococci.

SUMMARY

1. Incorporation of [^{14}C]amino acids into specific antibody and other γ-globulin *in vitro* by tissue slices, from immunized animals, occurred at a constant rate for a few hours after a short lag period provided that the cells were undamaged. After mild damage no incorporation into secreted proteins occurred, although insoluble tissue proteins still became labelled. Under our conditions, procedures which broke up the cells completely prevented incorporation of added [^{14}C]amino acids into specific protein. Evidence is given that incorporation of the ^{14}C-label into antibody is due to synthesis and secretion of antibody by the tissue slices.

2. By comparing the rates of incorporation of [^{14}C]glycine or valine *in vitro* by different tissues taken from rabbits immunized by different routes, it was possible to assess the relative contributions of these tissues to overall synthesis of specific antibody and other γ-globulin in the whole animal.

3. Rabbits immunized by intramuscular injection of ovalbumin in Freund's adjuvant mixture synthesized most of their antibody in the local granuloma, but, after further intravenous injection of alum-precipitated antigen, the spleen, lung and bone marrow became important sites of synthesis. When ovalbumin or pneumococci, or both, were injected intravenously the bone marrow, or in some animals the lungs, made most of the antibody. In rabbits immunized simultaneously with two different antigens, tissues varied considerably in their ability to synthesize each kind of antibody.

4. Tissues stimulated to produce specific antibody also produced increased amounts of other γ-globulin which was not identified as specific antibody.

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The Isolation and Identification of Phosphoethanolamine from the Urine of a case of Hypophosphatasia

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Rathbun (1948) described an unusual and previously unreported type of faulty bone development in a male child 3 weeks old. It was characterized by gross changes in long bones and skull, superficially similar to those in rickets, associated with a very low level of serum alkaline phosphatase. Post-mortem examination of bones, kidney and other organs revealed very low levels of alkaline phosphatase in these tissues also. Rathbun considered the low alkaline phosphatase to be the primary defect responsible for the clinical condition and called the new disease hypophosphatasia. Several other cases have subsequently been described (reviewed by Fraser, 1957), which suggests that the disease is not excessively rare but rather had not been previously recognized as a separate entity. McCance, Morrison & Dent (1955) and Fraser, Yendt & Christie (1955) independently and simultaneously reported finding phosphoethanolamine to be one of the main ninhydrin-reacting spots on the amino acid chromatograms of urine from two further cases in children. Phosphoethanolamine is not detected in normal urine by their chromatographic techniques. Cusworth & Dent (1956) confirmed these findings in six further child cases and one adult case of the disease and pointed out that although traces of phosphoethanolamine are occasionally found in the urine in other diseases, such as liver disease, it is then only a temporary phenomenon, whereas in hypophosphatasia the excretion of the compound is both large and constant. A small constant excretion of phosphoethanolamine is found in certain relatives of known cases of hypophosphatasia (see Fraser, 1957), suggesting an inborn condition. Cusworth & Dent (unpublished observations) have subsequently found the same large excretion of phosphoethanolamine in a further four child cases and two adult cases, bringing the total number of cases examined in this department to 13 and suggesting that excretion of the compound is to be considered as an essential feature of the disease.

The identification of phosphoethanolamine was in each case based on two-way chromatograms for urine amino acids and as such could be only tentative. Although identical chromatographic behaviour with synthetic phosphoethanolamine in several solvents and also by paper ionophoresis at various pH values was strongly suggestive, the ultimate proof of identity must, if possible, depend on isolation of the pure compound from the urine of a case of hypophosphatasia followed by its chemical analysis and study of physical properties. This has now been achieved.

EXPERIMENTAL

The patient who provided the material for this isolation was a woman of 41 years with a history of 'rickets' in childhood. She had been well until the last 4 years, when she began to get severe pains in her thighs and was found to have pathological fractures in both femora in the mid-shaft region. She was shown at the Royal Society of Medicine in 1953 by Dr G. H. Jennings as a case for diagnosis. Dr C. E. Dent, who saw her there, thought she had some unusual form of osteomalacia, but the chromatogram for urine amino acids then carried out in this department showed a large excretion of a substance considered to be phosphoethanolamine. The significance of this was not realized at the time. Subsequently, increased excretion of phosphoethanolamine was found in several child cases of hypophosphatasia and it was realized that this was a diagnostic feature of the disease. The original chromatogram was noted during a review of previous work in the department and the possibility that the adult patient might be a case of this disease was therefore considered. This was fully confirmed after detailed study in the Metabolic Ward, University College Hospital; no clinical details of these studies are yet published.

Urine collection. During the patient's stay in the Metabolic Ward, continuous 24 hr. urine collections were made. After portions had been removed for other investigations, the remainder was bulked and stored under toluene at 4°. A total of 32 l., corresponding to 21 days' output, was collected.

Preliminary isolation. In the early stages, the general procedure of Westall (1952) was followed. Two-way paper chromatograms (Dent, 1948) and high-voltage paper ionophoresis at pH 2 and 110 v/cm. (Gross, 1955) were used extensively throughout the isolation to check the amino acid content of the various fractions and the purity of the product. The 32 l. of urine was concentrated under reduced pressure in a circulating evaporator (Van Heyningen, 1949) to 2 l., 3 vol. of ethanol were added and the mixture was allowed to stand at 4° overnight and filtered. The filtrate contained phosphoethanolamine and amino acids and has

not yet been worked up. The precipitate also contained some amino acids and phosphoethanolamine, but the ratio of phosphoethanolamine to other amino acids was higher than in the filtrate, so the precipitate was dissolved in water and the residue removed, by centrifuging, and washed. The volume of extract and washings was 900 ml. and contained most of the phosphoethanolamine from the ethanol precipitate. The extract was treated with 5 g. of charcoal, boiled to remove CO_2, and the filtrate passed through a train of three columns of bed size 6 cm. (diam.) × 11 cm. and 3·5 cm. (diam.) × 13 cm. packed with Dowex 2 (× 8) anion-exchange resin (50–100 mesh; OH^- ion form). After washing with CO_2-free water, displacement was commenced with N-HCl and 250 ml. fractions were collected. Owing to the limited amount of resin available this step was carried out in three batches.

The amino acid-containing fractions from the Dowex 2 were diluted to 10 l. and passed through an 8 cm. × 33 cm. column of Zeo-Karb 215 cation-exchange resin (H^+ ion form). Most of the amino acids are retained by this resin but taurine and phosphoethanolamine pass through into the effluent. This was boiled for a short time to remove CO_2 and passed through the original set of Dowex 2 columns previously regenerated to the OH^- ion form. Displacement from the Dowex 2 was carried out with 0·2N-HCl and 25 ml. fractions were collected on a time-operated fraction collector. Phosphoethanolamine was very slightly retarded in coming off the column compared with taurine, but most fractions contained the two substances in equal amounts and no fraction contained one component free from the other.

Separation of phosphoethanolamine and taurine. These two substances are very similar in their behaviour on ion-exchange resins and consequently are difficult to separate on columns on the preparative scale, especially when they are present in equal amounts. Results of attempts to carry out the separation by eluting bands from one-way paper chromatograms and precipitating the barium salt of phosphoethanolamine in ethanol (cf. Plimmer & Burch, 1937; Kurtz & Luck, 1935) were not very encouraging, but electrodialysis seemed more promising. A three-compartment electrodialysis cell was set up as described by Campbell & Work (1952), the volume of each compartment being about 750 ml. A Permutit anion-exchange membrane (A 10) was used between anode and centre compartments and a cellophan membrane between cathode and centre compartments. A carbon rod was used for the cathode and a platinum wire for the anode. The fractions containing phosphoethanolamine and taurine were diluted to 500 ml., brought to pH 6·5 with 2N-NaOH and placed in the centre compartment. The cathode and anode compartments were filled with water and brought to pH 9 with aq. NH_3 soln. and to pH 3 with dil. H_2SO_4 respectively. A d.c. potential of 190v was applied, when a current of 40 mA passed which rose to 900 mA over the course of 2 hr. At this point two-thirds of the contents of the anode compartment were removed and replaced with water, when the current dropped to 400 mA and gradually fell to 100 mA during the next 4 hr., when the run was stopped. Analysis showed that the anode fractions contained phosphoethanolamine and a small amount of taurine. The fractions were put on a train of two columns of bed size 3·5 cm. × 12·5 cm. and 2 cm. × 6 cm. packed with the Dowex 2 resin used previously. Displacement was carried out with 0·1N-HCl and fractions were collected as before. The first three amino acid-containing fractions contained decreasing amounts of taurine and increasing amounts of phosphoethanolamine, the next three fractions contained large amounts of phosphoethanolamine, apparently free from other ninhydrin-reacting material, and the following fractions contained decreasing amounts of phosphoethanolamine and increasing amounts of chloride. The fractions containing only phosphoethanolamine were evaporated on a rotary evaporator, when crystals separated out. These were filtered off, twice recrystallized from 80% (v/v) methanol and once from water and dried at 100°. Yield 40 mg., which represents a very small portion of that present in the original urine.

Confirmation of identity. The isolated material showed identical behaviour with synthetic phosphoethanolamine (Clarke, Datta & Rabin, 1955) on paper chromatography in phenol–water (NH_3), lutidine–water (diethylamine) and butanol–pyridine–water, and on paper ionophoresis at pH 2 and pH 5·5, and in all cases gave only one spot on the paper. Its behaviour on ion-exchange resins and in the electrodialysis cell during the isolation was that expected of phosphoethanolamine. The m.p. of the isolated material was 242–243° (corr.), of synthetic phosphoethanolamine 243–244° (corr.) and mixed m.p. 241–242° (corr.) [Analysis (Organic Microanalytical Laboratory, Imperial College): C, 17·3; H, 5·8; N, 10·1; P, 22·1. Calc. for $C_2H_8O_4NP$: C, 17·0; H, 5·7; N, 9·9; P, 22·0%]. Infrared spectroscopy was carried out by Dr G. Wilkinson (King's College, London) who found that the isolated material and synthetic phosphoethanolamine gave identical absorption spectra. The isolated material was considered to be pure phosphoethanolamine.

DISCUSSION

The positive identification of phosphoethanolamine confirms the original suggestion of McCance *et al.* (1955) and Fraser *et al.* (1955) that it is excreted in excess in hypophosphatasia. Recently, Fraser's group in Toronto have succeeded in isolating a small amount of crystalline material from the urine of the physically normal father of a child case of hypophosphatasia. Examination of the melting point, X-ray-diffraction pattern, infrared spectrum and phosphorus:nitrogen ratio has led them to conclude that the material is phosphoethanolamine (Dr D. Fraser, personal communication). The finding of increased excretion of phosphoethanolamine in all cases so far examined suggests that it is part of the biochemical disturbance which occurs in hypophosphatasia. The original suggestion of Rathbun (1948) that the primary defect in this disease is a deficiency of alkaline phosphatase is still accepted and it is now considered to be genetically determined, so that this may be an inborn error of metabolism (Garrod, 1908) in which phosphoethanolamine is an intermediary metabolite excreted as a consequence of the metabolic block. The metabolism of other phosphate esters may also be abnormal but this has not so far been studied in detail; phosphoethanolamine has been

detected because it reacts with ninhydrin on amino acid chromatograms whereas most other phosphate esters do not. McCance, Fairweather, Barrett & Morrison (1956) have tentatively reported the excretion of adenosine monophosphate in one of their cases but this has not yet been confirmed (Dr T. A. J. Prankerd, personal communication).

Robison (1923) described the occurrence of phosphatase in ossifying cartilage and suggested that the hydrolysis of 'some phosphoric ester' might be a factor in bone formation. Since that time there has been much speculation as to the nature of the physiological substrate for phosphatase, and the role of this enzyme in bone formation is still uncertain (Bourne, 1956). It is tempting to link together the findings in hypophosphatasia of reduced phosphatase activity, phosphoethanolamine excretion and deranged bone growth and to suggest that phosphoethanolamine might be a substrate for phosphatase in bone formation. Phosphoethanolamine occurs abundantly in tissues (Colowick & Cori, 1939; Tallan, Moore & Stein, 1954), and may be concerned in phospholipid metabolism (Ansell & Dawson, 1952), but apart from this its function in the body is not known. It is not normally detected in plasma or urine and thus is probably usually an exclusively intracellular substance. Phosphoethanolamine is hydrolysed by alkaline phosphatase *in vitro* (McCance *et al.* 1956), but, owing to the low specificity of this enzyme, the fact that phosphoethanolamine can act as a substrate does not prove that it is a physiological substrate for phosphatase, so this can be no more than a tentative suggestion.

The exact significance of excretion of phosphoethanolamine in hypophosphatasia is not known, but from a practical point of view it can be of use in the diagnosis of this disease. Increased excretion of phosphoethanolamine is sometimes seen in liver disease but is then part of a generalized amino aciduria (Walshe, 1951); hypophosphatasia is the only condition so far encountered in which there is a gross excretion of phosphoethanolamine with no other change in the pattern of excretion of amino acids.

SUMMARY

Phosphoethanolamine has been isolated in pure form from the urine of an adult case of hypophosphatasia and identified by chemical and physical examination.

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Incorporation of Radioactive Phosphorus into the Ribonucleic Acid of Subfractions Derived from Guinea-Pig-Liver Microsomes

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It is now generally accepted that there is a particularly rapid protein turnover in the microsome fraction of liver in various animals. We have already shown (Simkin & Work, 1957*a*) that the microsome fraction of guinea-pig liver can be separated by successive extractions with solutions of varying ionic strength and pH into several distinct subfractions, each containing proteins with different turnover rates. Two of these subfractions, *B* and *C*, appeared to be ribonucleoproteins, and after injection of [^{14}C]amino acids into normal guinea pigs the protein components of one of these two fractions (*C*) became labelled more rapidly than the other. Since a considerable amount of evidence has accumulated recently which suggests that ribonucleic acid (RNA) plays a special role in protein synthesis (Brachet, 1955), it seemed desirable to compare RNA turnover with protein turnover in the various subfractions from liver microsomes.

In the experiments described here, radioactive orthophosphate was injected into guinea pigs which were killed at appropriate time intervals. The microsome fraction was isolated and subfractionated as described earlier and the rate of incorporation of ^{32}P into the RNA of the different microsome subfractions was measured. It was found that in subfraction *C*, which had the highest rate of protein turnover, the rate of incorporation of ^{32}P into RNA was below that observed for any other microsomal subfraction.

EXPERIMENTAL

Estimation of ribose. The orcinol method of Mejbaum (1939) was used.

Spectrophotometric estimation of ribonucleic acid. The RNA was estimated by the method of Ogur & Rosen (1950); it was assumed that, under these conditions, RNA had an $\epsilon(P)_{260}$ of 10 800. Optical density was measured with the Unicam SP. 500 spectrophotometer.

Estimation of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Animals. Guinea pigs (male and female) from the Institute stock were employed (body wt. 700–800 g.). The animals were starved overnight before injection, except in the 18 hr. experiment, in which they were starved only after injection.

Injection of ^{32}P. Inorganic phosphate ([^{32}P]orthophosphate) in isotonic saline (Radiochemical Centre, Amersham, Bucks) was injected intravenously. For each time point two animals were used, each being injected with 500 μc of ^{32}P (500 μg. of P).

Isolation of microsome fraction. The animals were killed after the appropriate time interval by a blow on the head. The livers were removed as rapidly as possible, chilled, minced, homogenized in 0·25 M-sucrose and the homogenate was centrifuged by the procedure of Simkin & Work (1957*a*) to isolate the microsome fraction. The temperature was maintained near to 0° throughout.

Fractionation of the microsome material. Fractionation was started in each case with the microsome pellet obtained from 36 g. of liver mince. The pellet was washed twice with glycerol (Simkin & Work, 1957*a*) and the microsome material was then suspended by homogenizing in about 10 ml. of 0·15 M-NaCl; 1 ml. samples were taken for (*a*) protein estimation and (*b*) isolation of RNA, and the remaining material was fractionated by the method of Simkin & Work (1957*b*) to give subfractions A_1, A_2, B_1 (referred to subsequently as *B*), B_2, *C*, *D* and *E* (about 12 ml. of extractant was used for each of the fractions A_1, *B*, *C*, *D* and *E* and 24 ml. for A_2 and B_2). Samples (1 ml.) were taken from each fraction for (*a*) protein estimation and (*b*) determination of ultraviolet-absorption spectrum, and the remaining material was used for the isolation of RNA. The RNA of fractions A_1, A_2 and B_2 was estimated only in one (18 hr.) experiment; at most, only a trace of RNA was detected, which is in accord with earlier observations (Simkin & Work, 1957*a*).

Isolation of nucleic acid from the whole microsome material and from the subfractions. Each fraction was mixed at 0° with 30% (w/v) trichloroacetic acid (TCA) to give a final TCA concentration of 5%. The precipitate was collected by centrifuging and washed once with 5% TCA at 0°. Samples of the combined TCA supernatants were used for estimation of ribose; this ribose is referred to as the 'TCA-soluble ribose'. The TCA-insoluble material was washed two or three times with 5% (w/v) NaCl in the cold, and then, to remove lipids, twice each with ethanol, acetone, ethanol–ether (3:1, v/v) and finally once with ether. In the 1 hr. experiment, ribose estimations were made on the combined NaCl washes and on the combined organic solvents' washes; the sum of these two values has been termed 'ribose in washes'. RNA was extracted from the fat-free residue by three successive 1 hr. extractions at 100°, 1 ml. of 10% (w/v) NaCl being used for each extraction. Since some RNA remained unextracted by this treatment, the protein residue was digested with 2 ml. of M-perchloric acid (PCA) overnight at room temperature (cf. Ogur & Rosen, 1950) and ribose was estimated in the digest; this is referred to as the 'PCA-soluble ribose'. The 10% NaCl extracts were made up to 3·5 ml. and ribose was estimated on 0·2 ml. samples after perchloric acid digestion; this is referred to as the '10% NaCl soluble ribose'. As a check on

the above method, RNA was also estimated spectrophotometrically on 0·2 ml. samples of the NaCl extract from whole microsomes (*M*) and from fraction *B* (6 hr. experiment); the values were found to be 90 and 96% respectively of those obtained by the orcinol method. Ribonucleic acid was precipitated from the remainder of the NaCl extracts by addition of 7 ml. of ethanol, and was collected after leaving for 16 hr. at −4°. The precipitate was washed once each with ethanol and ether at 0°. In the 1, 6 and 18 hr. experiments, the combined ethanol and ether supernatants were evaporated to dryness and ribose estimations were made on the aqueous extract of the residue; this has been designated the 'ethanol-soluble ribose'.

Determination of radioactivity. The samples of RNA were dissolved in water, plated on aluminium planchets and the radioactivity of the samples was measured in a thin-window Geiger counter; in the 18 hr. experiment only a portion of the aqueous solution was plated. The amount of RNA on the planchet was obtained (except in the 30 min. experiment) by subtracting the amount of RNA left unprecipitated by ethanol from the amount of RNA in 10% NaCl extracts (method I). For conversion of ribose values into RNA values a factor of 4·38 was used; this factor is based on the nucleotide ratios of rabbit-liver microsomes (with adenylic acid as 1·0; guanylic acid = 1·58; cytidylic acid = 1·54; uridylic acid = 1·03) reported by Crosbie, Smellie & Davidson (1953). In the 30 min. and 1 hr. experiments, after counting, the sample was washed off the planchet with 0·01 N-NaOH (it was not found possible to remove all the radioactivity from the planchets by water), and RNA was estimated spectrophotometrically. (Method II.) In the 1 hr. experiment where both methods, I and II, were used for the subfractions *B* and *C*, the quantities of RNA as determined by the former method were 98 and 94% respectively of the values obtained by the latter method.

Separation of individual nucleotides by electrophoresis. The method used was essentially that of Davidson & Smellie (1952*a*). Since the amount of nucleic acid available from each fraction was small, it was found necessary to dilute it with RNA of known composition before hydrolysis. The dried sample of nucleic acid from fraction *B*, *C* or *D* (18 hr. experiment) was dissolved in 0·5 ml. of a stock solution of yeast RNA (nucleotide composition with adenylic acid as 1·0: guanylic acid = 1·17, cytidylic acid = 0·70; uridylic acid = 0·94) in water (568 μg. of RNA or 55 μg. of RNA-P/ml.); 0·1 ml. of the mixture was used for determination of specific activity as already described, and the rest was hydrolysed with 0·4 ml. of 0·6 N-KOH for 22 hr. at 37°. The pH of the solution was adjusted to 4·0 by the addition of the calculated quantity of perchloric acid and the $KClO_4$ removed. A measured volume (0·2–0·4 ml.) of this solution was submitted to electrophoresis for 8 hr. on Whatman no. 3 MM paper (57 cm. × 33 cm.; previously washed free of ultraviolet-absorbing material by elution with 0·2 N-HCl in a chromatography chamber for 48 hr.), an apparatus based on that of Kunkel & Tiselius (1951) being used. Citrate buffer of pH 3·5 was used, with a potential difference of 550 v and a gradient of 10 v/cm. (cf. Davidson & Smellie, 1952*a*). Nucleotides were located by ultraviolet photography; the spots were eluted with 0·02 N-HCl (5 ml.) and the nucleotides estimated by measurement of optical density at 260 mμ (the washed-paper blank, extracted in the same way, gave $E^{260}_{1\,cm.} = 0{\cdot}0033$/cm.^{2} of the paper). To check the identity of each nucleotide, absorbance ratios were determined at the following wavelengths, 250/260, 270/260 and 290/260 mμ; these agreed within experimental error with those given by Beaven, Holiday & Johnson (1955). The material eluted from each spot was then plated on an aluminium planchet and the radioactivity was measured. Good separation of nucleotides was obtained with 0·3–0·4 μg. of nucleotide P, and a migration time of 8 hr. Relative mobilities were (taking uridine monophosphate = 1·0): cytidine monophosphate 0·07; adenosine monophosphate 0·23; guanosine monophosphate 0·78. Davidson & Smellie (1952*a*) give somewhat different mobility values, but they used the Durrum (1950) open-strip type of electrophoresis apparatus.

Incorporation of ^{32}P into microsomal ribonucleic acid in a cell-free system. The livers from two guinea pigs were minced and homogenized in 0·25 M-sucrose under the conditions described by Simkin & Work (1957*b*). The nuclei and mitochondria were removed and the microsome-cell-sap fraction (7·5 ml., equivalent to 3 g. of liver) was incubated with the mixture of salts and 3-phosphoglycerate used earlier in experiments on amino acid incorporation (Simkin & Work, 1957*b*), and with either 25 μc or 125 μc of ^{32}P. Incubation was carried out at 37° in an atmosphere of $N_2 + CO_2$ (95:5). At appropriate times, the reaction was stopped by the addition of 15 ml. of an ice-cold solution containing sodium phosphate buffer (mM, pH 7·4) and sucrose (0·25 M). The cold suspension was centrifuged at $g_{av.}$ 78 000 as described by Simkin & Work (1957*b*) and RNA was isolated from the unwashed microsome pellet by the procedure described earlier. In this experiment the microsome material was not separated into individual subfractions.

RESULTS

Incorporation of ^{32}P into the ribonucleic acid of liver microsomes in vivo

The rate of incorporation of ^{32}P into the RNA of liver-microsome material was rather low and it was not possible to follow incorporation over the same time scale as was used earlier in studying protein turnover (Simkin & Work, 1957*a*). As can be seen from Table 1, the radioactivity of the RNA from the liver-microsome subfractions, isolated as

Table 1. *Specific radioactivity of ribonucleic acid isolated from microsomes of guinea-pig liver and from subfractions derived therefrom*

Animals were killed at various time intervals after the injection of [^{32}P]phosphate. *M* represents the whole microsome fraction; *B*, *C*, *D* and *E* are the subfractions derived from *M*. For method of fractionation see text. Specific activity is expressed as counts/min./mg. of RNA.

Time (hr.)	Specific activity of RNA				
	M	*B*	*C*	*D*	*E*
0·5	31	19	8	106	250
1·0	64	40	25	168	—
6·0	2240	1680	1750	2860	3580
18·0	—	4630	3900	5200	7210

described in the Experimental section, increased with time, but with some initial lag. The initial rate of uptake into the nucleoprotein fraction *C* was notably slower than that observed for other subfractions.

It seemed likely that the material extracted into 10% NaCl and precipitated by ethanol was entirely RNA, particularly since estimation by ultraviolet spectroscopy after perchloric acid digestion gave values within 6% of those obtained with the ribose method, but since with small quantities of RNA slight contamination with other radioactive phosphorus compounds might vitiate the results (cf. Davidson, Frazer & Hutchison, 1951; Davidson & Smellie, 1952*b*), the specific activity of individual nucleotides obtained by hydrolysis of RNA was also determined. This could not be done for the materials isolated from animals killed at short time intervals after injection, since the combination of low specific activity and small quantity of RNA would have reduced the specific-activity values for individual nucleotides to insignificant levels.

Satisfactory results were obtained with the RNA from subfractions of microsomes in the 18 hr. experiment. In this experiment, the RNA precipitated by ethanol after extraction into 10% NaCl (subfractions *B*, *C* and *D*) was diluted with yeast RNA and hydrolysed as described in the Experimental section. The specific radioactivities of the individual nucleotides, separated by ionophoresis on paper, are given in Table 2. The recoveries of nucleotide from fractions *B*, *C* and *D* were 72, 79 and 86% respectively, which correspond well with the radioactivity recoveries of 70, 83 and 94% (the recoveries are not quantitative because the area of paper occupied by nucleotide is not always clearly enough defined to permit exact delineation of the spot for elution). The results (Table 2) showed that the RNA isolated by extraction with 10% NaCl and precipitation with ethanol was free from radioactive contaminants and that subfraction *D* did indeed become more rapidly labelled than either subfraction *B* or *C*. Subfraction *E* did not contain enough RNA to permit separation of individual nucleotides, but since no contamination was detected in fraction *D*, it seems reasonable to assume that the RNA from fraction *E* was also free of radioactive contaminants.

Although RNA free from phosphorus contaminants could be isolated from the lipid-free microsome subfractions by extraction with hot NaCl soln. followed by precipitation with ethanol, there were considerable losses during isolation, particularly for subfractions *D* and *E*, due either to incomplete extraction or to incomplete precipitation. In these subfractions, a considerable amount of ribose-containing material, presumably obtained by degradation of RNA, was also found in the TCA

Table 2. *Distribution of radioactivity between different nucleotides obtained by hydrolysis of ribonucleic acid labelled with* ^{32}P

RNA was isolated from the subfractions *B*, *C* and *D* (obtained from the 18 hr. expt.) as described in the text. It was mixed with carrier yeast RNA, hydrolysed with alkali and the individual nucleotides were separated by paper electrophoresis as described in the text. Specific activity is expressed as counts/min./μmole of P.

Fraction	Specific activity of RNA	Specific activity of nucleotides*				Recovery of nucleotides† (%)	Recovery of radioactivity† (%)
		Ad	Gu	Cy	Ur		
B	1495	1535	1615	1290	1240	72	70
C	1268	1900	1670	965	1160	79	83
D	1685	2240	2005	1565	1510	86	94

* Ad, adenylic acid; Gu, guanylic acid; Cy, cytidylic acid; Ur, uridylic acid.

† These figures are the sum of the values for the four nucleotides and are expressed as percent of the calculated total nucleotide material or total radioactivity taken for electrophoretic separation.

Table 3. *Ribose content of different subfractions obtained from the microsome material of guinea-pig liver*

For method of fractionation of microsomes and of obtaining the various ribose-containing fractions from each microsomal subfraction, see text. All values are given as the mean of the four experiments, and are followed by the standard deviation. In each experiment 36 g. of liver mince was used.

Microsomal subfraction	10% NaCl soluble ribose (μg.)	TCA-soluble ribose (μg.)	Ribose in washes* (μg.)	PCA-soluble ribose (μg.)	Ethanol-soluble ribose (μg.)
B	533±75	24±3	23	91±13	27±8
C	191±29	29±3	9	32±8	15±5
D	43±10	23±5	7	41±9	14±8
E	16±4	9±2	9	12±5	11±3
$A_1+A_2+B_2$*	75	—	—	15	41

* Estimated in one experiment only.

supernatant and subsequent washes after the initial precipitation. The yield of RNA in the 10% NaCl extracts, and the various losses which occurred during isolation and purification of the RNA, are listed in Table 3. It is obvious that losses increased as the amount of RNA in the fraction decreased. Such losses seem unavoidable if reasonably pure samples of RNA are to be obtained. There were also quite substantial variations in the amounts of RNA obtained in different experiments as shown by the standard deviation values given in Table 3. This seemed to be due to normal biological variations which might be expected in a cell component such as RNA, which fluctuates with the nutritional state of the animal (Wikramanayake, Heagy & Munro, 1953). In the experiments reported here, subfraction *B* had the highest RNA content and the highest RNA:protein ratio (the average RNA:protein ratios for fractions *B*, *C*, *D* and *E* were 0·56, 0·32, 0·048 and 0·0088 respectively). In earlier experiments, the RNA:protein ratio for fraction *B* was sometimes lower than that for fraction *C* (Simkin & Work, 1957*a*). This variability does not appear to affect the pattern of protein labelling in the liver-microsome subfractions (Simkin & Work, 1957*b*).

Incorporation of ^{32}P *into microsomal ribonucleic acid in a cell-free system*

When a microsome–cell-sap preparation was incubated with radioactive phosphate and an energy source (3-phosphoglycerate), there was significant incorporation of ^{32}P into the RNA of the microsome material. The results of experiments with two different levels of ^{32}P are given in Table 4. There was an increase in the specific radio-activity of microsomal RNA for at least 30 min., and this was accompanied by a steady loss of RNA from the microsome material as reported by Simkin & Work (1957*b*).

Since under similar conditions to those used in this investigation, uptake of radioactive amino acids ceases rather abruptly after about 20 min. incubation, it seemed unlikely that the two processes, amino acid incorporation and ^{32}P incorporation, were directly related and further experiments were not performed.

Table 4. *Specific radioactivity of ribonucleic acid from guinea-pig-liver microsomes incubated* in vitro *for different time periods*

The microsome–cell-sap preparation was incubated under conditions described in the text. In Expts. 1 and 2, the amount of radioactivity in the incubation mixture for each time point was 25 and 125 μc of ^{32}P respectively.

Time (min.)	Specific activity (counts/min./mg. of RNA) Expt. 1	Expt. 2
0	21	86
15	46	187
30	101	392

DISCUSSION

Most cells which are capable of rapid protein synthesis are rich in ribonucleic acid, and, usually, protein synthesis and RNA synthesis seem to run in parallel during growth (Brachet, 1955). It is known also that exposure to ribonuclease frequently destroys the capacity of cells to synthesize protein (Brachet, 1955), and that removal of RNA from subcellular particles, either by exposure to ribonuclease or by extraction with sodium chloride, inhibits uptake of radioactive amino acids into proteins of these particles (Allfrey, Daly & Mirsky, 1953; Gale & Folkes, 1955; Marmur, Nisman & Hirsch, 1955). Therefore when we found (Simkin & Work, 1957*a*) that two ribonucleoprotein fractions could be obtained from guinea-pig-liver microsomes which differed considerably in their rate of protein turnover, it became a matter of considerable interest to determine the rate of RNA turnover in the same two fractions.

There is, in our results, no suggestion that RNA turnover runs parallel to protein turnover. Indeed, assuming that rates of uptake of radioactive amino acid and of radioactive phosphorus can be regarded as indices of the rates of protein and nucleic acid synthesis, it appears more likely that there is an inverse relationship between the rate of protein turnover and that of RNA. All experiments involving a single injection of isotope are complicated by rapid changes in radioactivity of precursor pools and by the possibility of intracellular-permeability barriers (cf. Reiner, 1953; Siminovitch & Graham, 1956), but since, in the present investigation, the ribonucleoprotein fractions are derived from microsome material, such difficulties are unlikely to vitiate results.

There is, in the literature, additional evidence consistent with the assumption of an inverse relationship between RNA turnover and protein turnover. Thus Hultin (1955), who first showed in 1950 that protein turnover is particularly rapid in liver microsomes, has emphasized that the RNA of soluble nucleoproteins of the cell sap has a higher rate of turnover than the microsomal RNA. Shigeura & Chargaff (1957) have recently fractionated microsomal material by a different method from that employed by us and have also found that a ribonucleoprotein fraction with a high protein-turnover rate had a particularly low rate of turnover of RNA. Smellie & Davidson (1956), working with several different ^{14}C-labelled precursors, have confirmed earlier work showing that cytoplasmic

RNA has a lower turnover rate than nuclear RNA, whereas for proteins the reverse is true. The results of recent elegant experiments of Clark, Naismith & Munro (1957), on the effect of protein starvation and protein feeding upon the rate of labelling of rat-liver RNA, point in the same direction. They found that RNA was stabilized by conditions favouring protein synthesis. It is thus clear that the results of the present investigation are in line with much other evidence obtained with mammalian cells. In each case, however, it is the rate of turnover of undefined trichloroacetic acid-precipitable protein which is being measured and where, as in micro-organisms, it has been possible to follow synthesis of a single induced enzyme, very different results have been obtained. Thus Pardee (1955), Gale (1956) and Spiegelman (1956) have all found that induced-enzyme formation was necessarily accompanied by synthesis of RNA. It is possible that simultaneous synthesis of RNA and protein is required only where new protein structures (e.g. induced enzymes) are being formed. In other cases of protein synthesis, as for example in the phenomenon of balanced synthesis and breakdown (protein turnover) in mature non-dividing mammalian cells, there may well exist an inverse relationship between the syntheses of protein and RNA. A precise explanation of this apparent contradiction may have to await elucidation of the exact role of RNA in protein synthesis.

SUMMARY

1. Guinea pigs were killed at various time intervals after intravenous injection of [^{32}P]phosphate. The liver-microsome fraction was subfractionated by the method of Simkin & Work (1957*b*). The ribonucleic acid of the various subfractions was isolated and the specific radioactivity of the nucleic acid from each subfraction was determined.

2. In one experiment, the ribonucleic acid isolated from three subfractions was hydrolysed to mononucleotides and their specific radioactivity was determined. On the basis of the results it was concluded that the ribonucleic acid was free from other ^{32}P-containing contaminants.

3. Incorporation of ^{32}P into the ribonucleic acid of the various microsome subfractions occurred at different rates. The ribonucleoprotein subfraction *C* had the lowest rate of ribonucleic acid turnover, although it has the highest rate of protein turnover.

4. These results are discussed in relation to other work on ribonucleic acid turnover during protein synthesis.

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Inhibition of Citrate Oxidation by Glyoxylate in Rat-Liver Homogenates

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Glyoxylic acid is known to inhibit the oxygen uptake of various respiring-tissue suspensions. The cause of this inhibition was attributed by Kleinzeller (1943) to a specific inhibition of the decarboxylative reactions, as it appeared in his experiments that the inhibition of decarboxylation of pyruvate by macerated yeast juice, and other tissue suspensions, increased with increasing concentration of glyoxylate. Since in our Laboratory, in the course of a study on the synthesis of glycine from glyoxylic acid (D'Abramo & Tomasos, 1955, 1956), it was found that the addition of different concentrations of glyoxylate to tissue suspensions produced varying inhibition of oxygen uptake, we investigated the mechanism of the inhibition produced by glyoxylate by studying its effect on the oxidation of components of the tricarboxylic acid cycle.

For this purpose, small amounts of glyoxylate were added to rat-liver homogenates incubated in the presence of citrate, *cis*-aconitate, *iso*citrate, α-oxoglutarate, succinate, fumarate, oxaloacetate, malate or pyruvate. The oxygen uptake was followed manometrically and citrate was determined at the end of the incubation period. The results showed that whereas 0·001 M-glyoxylate did not affect the endogenous respiration, it produced a small inhibition of the rate of oxygen uptake in the presence of almost all the citric acid cycle intermediates tested, and caused a small parallel increase of the citrate content of the tissue suspension. Both the inhibition of the oxidation, and the accumulation of citrate, reached very high values only when oxaloacetate was the substrate.

EXPERIMENTAL

Substrates. The following substrates were tested as acids neutralized to pH 7 at the start of the experiment: citric (Merck, Darmstadt, Germany); DL-*iso*citric (Sigma Chemical Co., St Louis, Mo., U.S.A.); α-oxoglutaric (General Biochemical Inc., Ohio, U.S.A.); succinic, pyruvic and fumaric (Eastman Kodak Organic Chemicals, Rochester, U.S.A.); malic (Bayer, Leverkusen, Germany); oxaloacetic, *cis*-aconitic and glyoxylic (Light and Co. Ltd. Colnbrook, Bucks). The purity of glyoxylate was tested by the chromatographic analysis according to Cavallini & Frontali (1954), which showed the two characteristic spots of glyoxylate. No oxalate was contained in our preparation, as shown by the absence of turbidity after the addition of a few drops of a saturated $CaCl_2$ solution.

Homogenates and incubation mixtures. Rat-liver homogenates were prepared by homogenizing (in an ice-cold glass Potter homogenizer) small pieces of liver with a phosphate–saline solution made from 27 ml. of the Krebs's phosphate–saline (Krebs, 1950), free from Ca^{2+} ions; 3 ml. of 0·1 M-phosphate buffer, pH 7·4, and 1 ml. of 0·1 M-$MgCl_2$. A volume (3 ml.) of this suspension, containing approx. 0·4 g. of homogenized tissue, was pipetted into Warburg vessels (about 20 ml. capacity) at 0°, containing 0·01 M-NaF and different concentrations (generally 0·002 M, corresponding to 8 μmoles/flask) of substrate, in the presence or absence of 0·001 M-glyoxylic acid (4 μmoles) neutralized immediately before use. The final volume of the incubation mixture was always 4 ml., giving a dilution of the homogenate of 1:10. Fluoride was added to the reaction mixture because preliminary experiments showed that the rate of citrate accumulation increased in the presence of this substance. The gas phase was O_2 and the time of incubation was 60 min. at 38°, unless otherwise indicated. The enzymic activity was stopped by adding either 0·5 ml. of 30% (w/v) trichloroacetic acid, or 2 ml. of 5% (w/v) tungstic acid, prepared immediately before use. The oxygen uptake was measured manometrically in the Warburg apparatus, CO_2 being absorbed on filter paper moistened with NaOH and placed in the centre well. Citrate was determined (*a*) after trichloroacetic acid deproteinization by the method of Pucher & Shermann (1936) as modified by Ruffo & D'Abramo (1953), or (*b*) after tungstate deproteinization by the method of Ettinger, Goldbaum & Smith (1952).

RESULTS

Effect of glyoxylate on the oxidation of the citric acid cycle intermediates

The effect of small concentrations (0·001 M) of glyoxylate added to many substrates of the citric acid cycle is reported in Table 1. Whereas addition of the low concentration of glyoxylate to the tissue suspension had little effect on the oxygen uptake (Table 1, Expt. 1), there was a small but constant inhibition of the oxygen uptake with the various substrates of the tricarboxylic acid cycle (Table 1, Expts, 2–5). At the same time a small increase in production of citrate was found in all the vessels at the end of the incubation period. Both these effects increased when oxaloacetate was the substrate (Table 1, Expt. 4). In this case it appeared that almost all the oxaloacetate added was recovered as citrate at the end of the incubation.

Effect of oxaloacetate and glyoxylate concentration on citrate accumulation

Since the greatest effect was observed when oxaloacetate was the substrate, an attempt was made in the following experiments to explain the mode of action of glyoxylate. The first point investigated was the effect of the addition of a small constant amount of glyoxylate together with increasing concentrations of oxaloacetate (Table 2).

The results of Expt. 1 (Table 2) demonstrated that the percentage inhibition of oxygen uptake was approximately constant, whereas the amount of extra citrate found at the end of the incubation

Table 1. *Effect of glyoxylate on oxygen uptake and citrate accumulation in rat-liver homogenates*

Rat-liver homogenate, 1:10; glyoxylate, 0·001 M; other substrates, 0·002 M; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Increase (μmoles)
1	None	18·7	—	0	—
	Glyoxylate	19·7	—	0	—
	Citrate	28·1	—	0·92	—
	Citrate + glyoxylate	26·2	6·7	1·56	0·64
2	*cis*-Aconitate	31·3	—	1·20	—
	cis-Aconitate + glyoxylate	30·6	2·2	1·70	0·50
	DL-*iso*Citrate	14·7	—	0·80	—
	DL-*iso*Citrate + glyoxylate	13·1	11·0	1·60	0·80
3	α-Oxoglutarate	28·0	—	1·12	—
	α-Oxoglutarate + glyoxylate	26·2	6·4	1·50	0·38
	Succinate	27·5	—	1·26	—
	Succinate + glyoxylate	25·1	8·7	3·00	1·74
4	Oxaloacetate	24·2	—	0·90	—
	Oxaloacetate + glyoxylate	13·5	44·0	10·20	9·30
	Pyruvate	28·2	—	1·90	—
	Pyruvate + glyoxylate	25·1	11·0	2·90	1·00
5	Fumarate	30·1	—	1·95	—
	Fumarate + glyoxylate	28·0	7·0	3·10	1·15
	Malate	26·4	—	2·44	—
	Malate + glyoxylate	24·0	8·6	3·04	0·60

Table 2. *Citrate accumulation at different concentrations of oxaloacetate*

Rat-liver homogenate, 1:10; glyoxylate, 0·001 M; oxaloacetate as indicated; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Increase (μmoles)
1	None	19·5	—	0·86	—
	Glyoxylate	19·6	—	0·85	—
	Oxaloacetate (0·001 M)	21·3	—	0·77	—
	Oxaloacetate + glyoxylate	10·1	51	4·65	3·88
	Oxaloacetate (0·002 M)	22·5	—	1·05	—
	Oxaloacetate + glyoxylate	11·5	51	9·10	8·05
	Oxaloacetate (0·003 M)	23·0	—	2·30	—
	Oxaloacetate + glyoxylate	12·8	46	10·90	8·60
	Oxaloacetate (0·004 M)	24·6	—	8·50	—
	Oxaloacetate + glyoxylate	13·0	48	15·60	7·10
2	None	19·9	—	0·60	—
	Glyoxylate	20·5	—	0·89	0·29
	Oxaloacetate (0·005 M)	29·5	—	7·62	—
	Oxaloacetate + glyoxylate	14·8	49	21·10	13·48
	Oxaloacetate (0·01 M)	30·1	—	10·50	—
	Oxaloacetate + glyoxylate	15·2	50	26·60	16·10

Table 3. *Accumulation of citrate at different concentrations of glyoxylate*

Rat-liver homogenate, 1:10; oxaloacetate, 0·002 M; glyoxylate as indicated; NaF, 0·01 M; pH 7·4; incubation, 60 min.; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Expt. no.	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Increase (μmoles)
1	None	19·7	—	0·61	—
	Oxaloacetate	21·0	—	0·92	0·31
	Glyoxylate (0·001 M)	20·1	0	0·92	—
	Oxaloacetate + glyoxylate	13·4	36	8·56	7·64
2	Glyoxylate (0·002 M)	12·3	40	2·40	—
	Oxaloacetate + glyoxylate	12·4	41	8·40	6·00
3	Glyoxylate (0·004 M)	9·8	52	1·80	—
	Oxaloacetate + glyoxylate	11·2	48	6·80	5·00

increased with oxaloacetate concentration up to 0·002 M and then remained constant. Moreover, in the experiments with glyoxylate (0·001 M) and increasing concentrations of oxaloacetate, the formation of citrate appeared to be roughly proportional to the oxaloacetate added. Expt. 2, with higher concentrations of oxaloacetate, confirmed the results on the inhibition of the oxygen uptake, but also showed that the citrate which accumulated was no longer proportional to the concentration of oxaloacetate added. Thus it appeared that the optimum concentration of oxaloacetate for testing the effect of glyoxylate on the accumulation of citrate was 0·002 M, where the synthesis of citrate without the inhibitor was practically nil and consequently the effect of the inhibitor was more evident.

The second point investigated was the effect of adding increasing amounts of glyoxylate to a fixed concentration (0·002 M) of oxaloacetate. The results (Table 3) showed that the amount of citrate found at the end of the incubation was not increased by raising the concentration of glyoxylate from 0·001 to 0·004 M, in contrast with the increase in the preceding experiments after the increase in oxaloacetate concentration (Table 2). The amount of citrate found was also here (Table 3) approximately equivalent to the oxaloacetate added. At the same time Expt. 1 (Table 3) confirmed that 0·001 M-glyoxylate did not affect the endogenous oxygen uptake of the liver, but produced a marked inhibition of the oxidation of oxaloacetate and a very marked accumulation of citrate. Moreover, in the control experiments (glyoxylate and liver without oxaloacetate) there was an increased production of citrate with an increase of glyoxylate concentration from 0·001 to 0·004 M. This citrate accumulation was probably due to the inhibition by glyoxylate of the oxidation of oxaloacetate either present in the liver or formed in the course of incubation. The parallel inhibition of oxygen uptake and citrate accumulation (Table 3, Expts. 2 and 3), which did not occur at the lowest glyoxylate concentration (Expt. 1), seems to confirm this possibility. The inhibition of oxygen uptake in these last experiments (2 and 3) was calculated by assuming a value of 19·5 μl./min. as basal rate (average of four control experiments reported in Tables 1–3).

Inhibition by glyoxylate of citrate oxidation

The results in Table 3, showing that the accumulation of citrate did not increase with increasing concentration of glyoxylate, suggested that the accumulation of citrate was due to an inhibition of its oxidation by glyoxylate rather than by an extra synthesis of citrate from oxaloacetate and glyoxylate. The experiments reported in Table 4, showing that the addition of 0·002 M-glyoxylate to a reaction mixture containing citrate completely prevented disappearance of citrate after the first 15 min., seem to confirm this possibility. It will be noticed that with citrate plus glyoxylate (0·002 M) at 15 and 30 min. the uptake of oxygen was in excess of that expected by the disappearance of citrate. This is also shown in the experiments reported in Table 6, and is probably due to endogenous substrates or to some glyoxylate being oxidized. However, 0·001 M-glyoxylate did not produce an accumulation of citrate, probably because, after part of the glyoxylate had been oxidized, the amount left was too low to cause inhibition.

These results, and the preceding ones showing that oxaloacetate was necessary for the maximal effect to be obtained, lead us to suppose that the inhibition of citrate oxidation may possibly be produced by a new inhibitor formed by a condensation between glyoxylate and oxaloacetate. Oxaloacetate, in the experiments of Tables 4 and 6, could have been formed from citrate during its initial oxidation. In this case the addition of oxaloacetate, together with glyoxylate, should produce a larger inhibition of the oxidation of citrate than that given by glyoxylate alone. Table 5 compares the

Table 4. *Effect of glyoxylate on citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002 M; glyoxylate as indicated; NaF, 0·01 M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Decrease (μmoles)
0	Citrate + glyoxylate (0·002 M)	—	—	8·5	—
15	Citrate	34·8	—	3·0	5·5
15	Citrate + glyoxylate (0·001 M)	35·0	0	3·1	5·4
15	Citrate + glyoxylate (0·002 M)	34·3	2	7·3	1·2
30	Citrate	32·6	—	1·2	7·3
30	Citrate + glyoxylate (0·001 M)	32·1	1	1·3	7·2
30	Citrate + glyoxylate (0·002 M)	28·0	14	7·5	1·0
60	Citrate	29·6	—	0·1	8·4
60	Citrate + glyoxylate (0·001 M)	30·1	0	0·4	8·1
60	Citrate + glyoxylate (0·002 M)	17·1	43	7·3	1·2

Table 5. *Effect of addition of oxaloacetate plus glyoxylate on citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002 M; oxaloacetate, 0·002 M; glyoxylate, 0·001 M; NaF, 0·01 M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Δ (μmoles)
0	Citrate + oxaloacetate + glyoxylate	—	—	7·9	—
15	Citrate + oxaloacetate	38·1	—	4·5	−3·4
15	Citrate + glyoxylate	37·7	1	4·5	−3·4
15	Citrate + oxaloacetate + glyoxylate	24·6	36	13·5	+5·6
30	Citrate + oxaloacetate	34·7	—	3·9	−4·0
30	Citrate + glyoxylate	31·6	9	3·9	−4·0
30	Citrate + oxaloacetate + glyoxylate	20·0	42	17·2	+9·3

Table 6. *Effect of glyoxylate on the earlier stages of citrate oxidation*

Rat-liver homogenate, 1:10; citrate, 0·002 M; glyoxylate, 0·002 M; NaF, 0·01 M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate Found (μmoles)	Citrate Decrease (μmoles)
0	Citrate + glyoxylate	—	—	8·85	—
5	Citrate	—	—	7·51	1·34
5	Citrate + glyoxylate	—	—	7·51	1·34
10	Citrate	—	—	6·00	2·85
10	Citrate + glyoxylate	—	—	6·20	2·60
15	Citrate	29·2	—	4·40	4·45
15	Citrate + glyoxylate	27·8	5	6·51	2·34
20	Citrate	30·0	—	4·10	4·75
20	Citrate + glyoxylate	28·2	6	8·35	0·50

inhibition of citrate oxidation, caused by glyoxylate (0·001 M) and oxaloacetate (0·002 M) with that obtained with glyoxylate alone. The glyoxylate or glyoxylate and oxaloacetate was added from the side arm to the main chamber at the beginning of the incubation. Under these conditions, the inhibition of the citrate oxidation was much larger than that observed in the preceding experiment at the corresponding time, and greater formation of citrate occurred; after incubation for 30 min. this roughly corresponded with the transformation of all the added oxaloacetate into citrate.

Formation of an inhibitor during the incubation of glyoxylate and oxaloacetate

The previous results lead us to suppose that both the inhibition of the oxygen uptake and the accumulation of citrate could be produced by a

Table 7. *Effect of oxaloacetate plus glyoxylate on citrate oxidation at the earlier stages of incubation*

Rat-liver homogenate, 1:10; citrate, 0·002 M; oxaloacetate, 0·001 M; glyoxylate, 0·001 M; NaF, 0·01 M; pH 7·4; temp., 38°; final vol., 4 ml.; gas phase, O_2.

Time (min.)	Additions	O_2 uptake (μl./min.)	Inhibition (%)	Citrate	
				Found (μmoles)	Δ (μmoles)
0	Citrate + oxaloacetate + glyoxylate	—	—	8·4	—
5	Citrate + oxaloacetate	—	—	5·5	−2·9
5	Citrate + oxaloacetate + glyoxylate	—	—	10·6	+2·2
10	Citrate + oxaloacetate	41·4	—	5·0	−3·4
10	Citrate + oxaloacetate + glyoxylate	26·0	37	12·4	+4·0
15	Citrate + oxaloacetate	41·0	—	4·4	−4·0
15	Citrate + oxaloacetate + glyoxylate	25·8	34	12·5	+4·1
20	Citrate + oxaloacetate	43·2	—	4·0	−4·4
20	Citrate + oxaloacetate + glyoxylate	22·2	49	12·4	+4·0

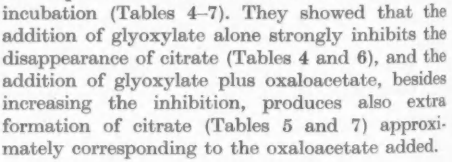
substance formed during the incubation of glyoxylate with oxaloacetate. In this case, after the addition of glyoxylate alone there would be an initial lag period before the inhibition of the oxidation of citrate occurred, since for the inhibition the formation of oxaloacetate from citrate would be necessary. This idea is in agreement with the results in Table 6, where citrate (0·002 M) was incubated for 5, 10, 15 or 20 min. in the presence or absence of glyoxylate (0·002 M). They show that glyoxylate did not interfere with citrate oxidation during incubation for 5 or 10 min., but inhibition started after 15 min. and only after 20 min. was it almost complete. This gradual appearance of inhibition did not occur in the next experiment (Table 7). Here the inhibition occurred immediately because glyoxylate (0·001 M final concentration in vessel) and oxaloacetate (0·001 M final concentration in vessel) were added together from the side arm at the beginning of the incubation. A very high inhibition of citrate oxidation occurred even after incubation for 5 min., the maximum value being already reached after 10 min. and remaining constant until 20 min. Moreover, it appeared that the citrate formed exceeded the value of citrate initially present in the flask (8 μmoles) by an amount corresponding approximately to the oxaloacetate added (4 μmoles).

DISCUSSION

The results presented in this paper confirm that glyoxylate is an inhibitor of tissue oxidations, and strongly indicate that one of the main points of action of this substance is the oxidation of citrate.

The finding which showed that the depression of the uptake of oxygen and the accumulation of citrate reached maximal rates only when glyoxylate and oxaloacetate were incubated together (Table 1), leads us to suppose that oxaloacetate under these conditions formed citrate, which was not further oxidized. However, the formation of citrate did not seem to be caused by the condensation of glyoxylate plus oxaloacetate, as might be supposed from the results of Campbell, Smith & Eagles (1953), Olson (1954) and Saz & Hillary (1956), who found that crude extracts of several micro-organisms synthesize citrate from glyoxylate plus succinate. Nor does the so-called 'glyoxylate bypass', recently discovered by Kornberg & Krebs (1957) in cell-free extracts of *Pseudomonas*, explain our observations. The observations that the formation of citrate (*a*) was always correlated with the inhibition of the oxygen uptake and (*b*) depended on increasing oxaloacetate concentration (Table 2), whereas it was practically independent of increase in the concentration of glyoxylate (Table 3), are against these possibilities. It seems, on the contrary, that both the depression of oxygen uptake and the formation of citrate can be explained by supposing that oxaloacetate may react in liver cells in two different ways: (*a*) with acetylcoenzyme A to form citrate; (*b*) with a small amount of glyoxylate to give rise to an inhibitor of citrate oxidation.

The formation of an inhibitor is in accord with the experiments with citrate added before the incubation (Tables 4–7). They showed that the addition of glyoxylate alone strongly inhibits the disappearance of citrate (Tables 4 and 6), and the addition of glyoxylate plus oxaloacetate, besides increasing the inhibition, produces also extra formation of citrate (Tables 5 and 7) approximately corresponding to the oxaloacetate added.



Oxalomalic acid

This inhibitory compound could well be a new tricarboxylic acid containing six carbon atoms, such as oxalomalic acid. It seems very likely that such a compound, even at very low concentrations, could compete with the enzymic systems related to citrate oxidation.

The existence of the inhibitory effect of glyoxylate on the tricarboxylic acid cycle appears to be of great interest if we consider that glyoxylate has recently been shown to take part in many metabolic transformations involving carbohydrates, fats and protein derivatives. The fact that small amounts of glyoxylate in the presence of oxaloacetate will inhibit citrate oxidation strongly indicates that in the cells under physiological conditions, glyoxylate, however formed, must be promptly removed to avoid the inhibitory effects described above. On the other hand, glyoxylate could inhibit the normal rate of oxidations and might be connected with the widely observed oxidative impairment of cancer tissues (see Warburg, 1956; Weinhouse, Warburg, Burk & Schadē, 1956; Ruffo, Capobianco & Adinolfi, 1956*a*, *b*), which has not yet been adequately explained.

SUMMARY

1. The effect of low concentrations (0·001 M) of glyoxylate on the oxidative rate of the main intermediates of the tricarboxylic acid cycle was investigated in respiring rat-liver homogenates.

2. The addition of glyoxylate had little effect on the endogenous oxygen uptake of the homogenate, but produced a small inhibition of the oxidative rate with citrate, D-*iso*citrate, *cis*-aconitate, α-oxoglutarate, succinate, fumarate, malate and pyruvate, and a small increase in the accumulation of citrate. A very marked inhibition of the uptake of oxygen, and a large accumulation of citrate, occurred when oxaloacetate was the substrate.

3. The oxidation of citrate was inhibited either by glyoxylate alone, after incubation for 20 min., or by glyoxylate plus oxaloacetate, after incubation for 5 min. Both these results indicate that oxaloacetate was necessary for obtaining maximal rates on inhibition.

4. The dependence of citrate accumulation on oxaloacetate and glyoxylate suggests that the two substances react together to form an inhibitor of citrate metabolism. This would account for both citrate accumulation and the depressed oxygen uptake.

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The Relationship between Catalase and Haemoglobin in Human Blood

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There is insufficient evidence so far to indicate the biological function of catalase, but there is indication that its presence in the living cell is protective against the toxic effects of hydrogen peroxide which may be produced as a by-product during the various oxidative enzymic reactions that occur in living organisms. (Keilin & Hartree, 1945).

Catalase, which like haemoglobin is a haematin–protein complex, is found principally in the red-blood cells and liver cells and is produced in the marrow and liver cells (Theorell, 1951). Its presence in plasma is probably due to haemolysis (Perlmann & Lipmann, 1947).

Interest in a possible role for hydrogen peroxide in the catabolism of haemoglobin was initiated by Bingold (1935), who found that haemoglobin could

be oxidized by hydrogen peroxide with resulting splitting of the tetrapyrrolic haematin ring into two dipyrrolic compounds, which then could be reduced to pink compounds—the pentdyopent reaction. It was later found, however, that this reaction could be obtained with other tetrapyrroles such as bile pigments.

Lemberg & Legge (1949) found that, under conditions approximating to those found in the circulating red cell, haemoglobin is converted into choleglobin (an iron-containing tetrapyrrole–globin complex with the tetrapyrrole ring modified) by coupled oxidation with ascorbic acid in the presence of oxygen. The ascorbic acid acts as a hydrogen donor, and an intermediary haemoglobin–hydrogen peroxide complex is formed. They also found that choleglobin formation in mammalian red cells is increased when the cells are haemolysed, and correlated this increase with a fall in catalase concentration that occurs with loss of stroma of the red cells.

In a review of some haemolytic mechanisms, Ponder (1951) has suggested that the presence of catalase in the red cell may be important in relation to red-cell ageing, since choleglobin-containing red cells are abnormally fragile, osmotically and mechanically. Like Lemberg & Legge, he considers it possible that catalase helps to preserve the red cell by reversing the change of haemoglobin to choleglobin at the intermediate haemoglobin–hydrogen peroxide stage, and quotes Foulkes & Lemberg (1949), who state that mammalian red cells rich in catalase have a longer life span than avian red cells, which have a low concentration of catalase. Keilin & Hartree (1945), however, have found that hydrogen peroxide which was being continuously generated in a system containing glucose oxidase produced small amounts of choleglobin and methaemoglobin from oxyhaemoglobin despite the presence of catalase.

Because of the common haematin prosthetic group of haemoglobin and blood catalase, their common distribution and common site of manufacture, and because of this possible protective effect of catalase for haemoglobin, it was decided to investigate the quantitative relationships between them in health and disease, especially anaemia.

Catalase acts by accelerating the decomposition of hydrogen peroxide into water and oxygen and its activity can therefore be estimated manometrically (e.g. Perlmann & Lipmann, 1947), or titrimetrically (e.g. Euler & Josephson, 1927; Bonnichsen, Chance & Theorell, 1947). In these methods, the velocity constants for the reactions were determined and used to estimate the activity of the catalase preparations by substitution in the formula Kat.f. $= k/$(g. of catalase protein in 50 ml. reaction mixture), where k is the reaction-velocity constant and Kat.f. (Euler & Josephson, 1927) is the measure of purity of the preparation. Herbert & Pinsent (1948) found that crystalline human-erythrocyte catalase had a Kat.f. value of 63 000.

Euler & Josephson used dilute solutions of catalase and hydrogen peroxide and took readings of the resulting reactions at 3 min. intervals for 12 min. The reaction-velocity constants of each reading were calculated. These decreased in a linear fashion with time, since catalase is progressively destroyed during the reaction, so that the results had to be extrapolated to zero time, when no catalase had yet been destroyed. Bonnichsen *et al.* modified this method, by increasing the speed of the reaction with much larger concentrations of the enzyme, and took several readings during a period of 1 min.; the reaction-velocity constant then did not vary with time.

Using a manometric method, Perlmann & Lipmann (1947) also determined the Kat.f. value for their enzyme preparation obtained from blood, and expressed their results as a percentage of a 'pure' preparation of erythrocyte catalase of Kat.f. value 43 000. Theorell (1951) and Beers & Sizer (1953) have criticized the manometric procedure as not suitable for kinetic studies of catalase.

In the above methods the authors had attempted to obviate the destructive effect of the substrate upon the enzyme. They used an excess of enzyme so that all the substrate would have been destroyed had the reactions gone to completion, and assumed these to be of the first order. The timing of the readings had to be accurate, so that the necessary manipulations became difficult if many samples were to be examined.

The method used in this study employs a different principle. The destructive effect of the substrate is utilized so that all the enzyme is destroyed before complete decomposition of the hydrogen peroxide is obtained. The reaction, which under these conditions is not of the first order, is allowed to proceed to completion so that time is no longer a critical factor and the residual hydrogen peroxide is estimated titrimetrically. It is shown that the amount of the substrate utilized is directly proportional to the initial enzyme concentration, and it is easy in this way to compare the activities of different samples. To satisfy these conditions, the concentration of the enzyme must be considerably less than hitherto used.

It was found that haemoglobin and catalase are present in the red cell in a fairly constant proportion, irrespective of the severity of anaemia.

MATERIALS

Apparatus. A water bath at 37° and a rack for tubes, flat-bottomed glass tubes (5·5 cm. × 1·5 cm.) and a Conway microburette fitted with a mixing air bubbler (i.e. a small

pump passing a stream of air bubbles through the sample being titrated to ensure rapid mixing of the samples and the titrating fluid) were used.

Reagents. Potassium permanganate soln. (0·1 N); 0·02 M-phosphate buffer, pH 6·8; 2 N-H_2SO_4. AnalaR hydrogen peroxide solution ('90–100 volumes') was used for preparation of the substrate solutions by dilution with the phosphate buffer.

All the glass-ware was washed in hot soapy water and rinsed in several changes of water before drying in a hot-air cupboard. The use of acid–dichromate cleansing solution was found to be unsatisfactory owing to the difficulty of removing all traces of this solution, which then reacted with the hydrogen peroxide and so gave false measurements.

The blood under investigation was collected by one of two methods: either by skin prick from the finger tip or lobe of the ear for immediate assay, or by venepuncture if there was likely to be any delay before it could be examined. In the latter case the blood was kept fluid with Wintrobe's oxalate mixture (Whitby & Britton, 1950) and showed no appreciable loss of activity even if stored up to 3 weeks at 4°.

Once the blood had been diluted to the desired concentration, it was necessary to determine its activity as quickly as possible because, after dilution, the catalase activity decreased at a rate of about 5%/hr.

METHODS

Haemoglobin estimation. The oxyhaemoglobin method described by Whitby & Britton (1950) was used except that 50 μl. of blood was diluted in 7 ml. of 0·04% (v/v) aq. NH_3 soln.

Whole-blood protein estimation. This was done with the micro-Kjeldahl–nesslerization method as described by Varley (1954).

Estimation of catalase activity. Whole blood (50 μl.) was laked in 7 ml. of 0·04% (v/v) aq. NH_3 soln. and the haemoglobin content estimated. The white cells, which contain peroxidase, were then removed by centrifuging. A solution containing 5 μg. of haemoglobin/ml. was then prepared by diluting with the buffer solution. Portions (2 ml.) were then placed in two tubes, which were transferred to a water bath at 37° for 5 min. to attain water-bath temperature. A volume (1 ml.) of 0·03 M-hydrogen peroxide soln., previously warmed to 37° in the water bath, was then added to each tube and also to two 'blank tubes' containing 2 ml. of phosphate buffer only. These 'blank tubes' were used to determine the initial amount of hydrogen peroxide added. The tubes were allowed to remain in the water bath for 75 min., when the reactions were complete. Sulphuric acid (2 ml. of 2 N soln.) was added to each tube and the residual hydrogen peroxide titrated with 0·1 N-$KMnO_4$ with a Conway microburette (1 ml. of 0·1 N-$KMnO_4 \equiv 560$ μl. of O_2 at s.t.p. derived from the H_2O_2 soln.). Because of the low concentrations of blood used, the amount of $KMnO_4$ reacting with the protein, or with the oxalate anti-coagulant, could be neglected.

The results were expressed as μl. of oxygen at s.t.p. evolved from the hydrogen peroxide during the reaction. This figure was obtained by converting the difference of the 'blank' and final titrations into volumes of oxygen, and was referred to as the number of units of catalase activity/10 μg. of haemoglobin.

To ensure that the loss of hydrogen peroxide that occurred was due to its breakdown into water and oxygen, and not to some other possible peroxidative or additive reaction, duplicate measurements were carried out on some of the blood samples with a manometric technique. The same quantities and dilutions of reagents were used in a Warburg manometric apparatus, and the total quantity of gas evolved, after the reaction had ceased, was estimated. Provided that the flasks were not shaken, the oxygen evolved was equivalent to the amount of hydrogen peroxide destroyed in the titrimetric method, and this was further confirmed by estimating the residual hydrogen peroxide in the manometric flasks.

During this experiment it was found that if the manometric flasks were shaken in the usual way, there was a considerable reduction in the amount of oxygen evolved and an equivalent reduction in the quantity of hydrogen peroxide destroyed. The effect of shaking on the reaction mixture is being further investigated, as it seems likely that this is the reason for the discrepancies observed in the manometric procedures by Theorell (1951) and Beers & Sizer (1953).

RESULTS

Fig. 1 shows that the reaction was completed in 60 min., although unchanged hydrogen peroxide was still present at this time; no further decomposition occurred even after 3 hr.

Fig. 2 shows that so long as there is incomplete breakdown of substrate at the end of the reaction, there is a linear relationship between blood dilutions, i.e. the catalase content, and the amount of hydrogen peroxide finally destroyed. Furthermore, there is a maximum quantity of substrate that can be decomposed by a given amount of catalase under these conditions.

From these results, it was found convenient to use 0·03 M-hydrogen peroxide solution, giving a final substrate concentration of 0·01 M, a final blood dilution of 10 μg. of haemoglobin/3 ml., and to allow at least 75 min. to elapse before estimating the residual hydrogen peroxide.

Repeated tests on one sample over several days showed an experimental error of just under 6%. Tests were also carried out at irregular intervals on one of the normal subjects, and the results always fell within a range of 10%, indicating a fairly constant figure for each subject.

It was desirable to calculate from the results obtained by this method the activity in terms of Kat.f., but since these were measured and calculated under different conditions from those of the present method, an analysis of the kinetics of the reactions taking place is necessary.

Curve *A* of Fig. 3 shows the logarithm of the concentration of undecomposed substrate plotted against time, from the results presented in Fig. 1. Curve *A* is not linear so that the overall reaction is not of the first order.

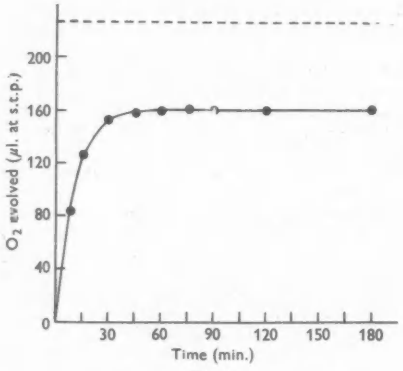

Fig. 1

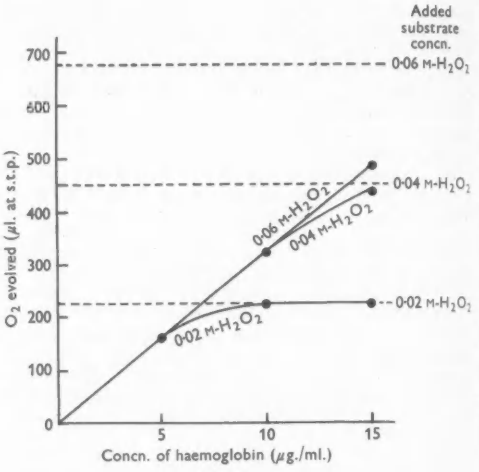

Fig. 2

Fig. 1. Reaction of blood catalase with excess of hydrogen peroxide. Results of a timed experiment with a series of identical blood and substrate samples. The specimen of blood was diluted to contain 5 μg. of haemoglobin/ml. and the substrate concentration added was 0·02 M (1 ml. of 0·02 M-hydrogen peroxide solution = 224 μl. of available oxygen at s.t.p.). The horizontal broken line represents the available oxygen added to the enzyme.

Fig. 2. This composite curve represents the results of three experiments, allowed to proceed to completion, with three concentrations of added substrate [0·02, 0·04 and 0·06 M-hydrogen peroxide solutions (i.e. 224, 448 and 672 μl. of oxygen at s.t.p. respectively)] and three concentrations of the same blood sample (5, 10 and 15 μg. of haemoglobin/ml.). The horizontal broken lines show the total available added oxygen for each experiment.

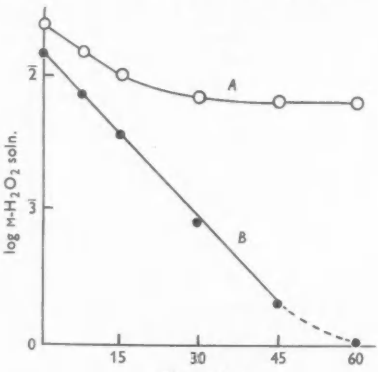

Fig. 3

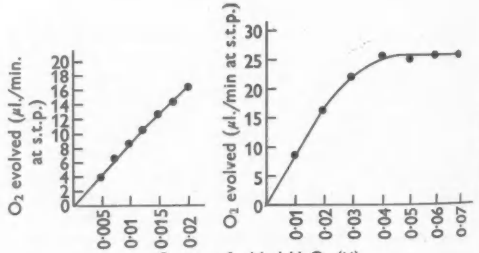

Fig. 4

Fig. 3. The logarithm of substrate concentration left at different times is plotted against time, and is derived from the results shown in Fig. 1. In curve *A*, the zero-time concentration of substrate is taken as the concentration of the added hydrogen peroxide. Curve *B* is constructed on the assumption that the zero-time concentration was the amount of substrate finally destroyed.

Fig. 4. Reaction velocities obtained from a series of experiments allowed to proceed for 3 min. before being stopped with 2 ml. of 2 N-sulphuric acid. The blood sample used for Figs. 1 and 3 was used and diluted to 5 μg. of haemoglobin/ml. The left-hand figure is a detail from the first part of the right-hand figure. With this particular blood sample the maximum added substrate concentration that could be fully decomposed was 0·014 M (i.e. an activity of 160 units).

It is clear that the overall reactions between catalase and hydrogen peroxide under the conditions of these experiments involves not only the decomposition of the substrate but also the destruction of the enzyme itself.

Suppose the enzyme is destroyed in a first-order reaction, i.e. $-\frac{dE}{dt}=k_4E$, where E is enzyme concentration at time t. Therefore

$$E=b\,e^{-k_4t}, \qquad (1)$$

where b is initial enzyme concentration. Now suppose that the enzyme reaction obeys the Michaelis & Menten (1913) law, i.e.

$$\frac{dx}{dt}=\frac{k_3E(a-x)}{K_m+(a-x)}, \qquad (2)$$

where a is initial substrate concentration, x is substrate decomposed at time t, K_m is the Michaelis constant.

Therefore $$\frac{dx}{dt}=\frac{k_3b\,e^{-k_4t}(a-x)}{K_m+(a-x)}$$

by substituting (1) in (2). Therefore

$$\int\frac{K_m+(a-x)}{(a-x)}\,dx=k_3b\int e^{-k_4t}\,dt,$$

i.e. $$x-K_m\ln(a-x)=\frac{-k_3}{k_4}b\,e^{-k_4t}+C.$$

Now, $x=0$ when $t=0$, so that C can be determined. Therefore

$$x+K_m\ln\frac{a}{a-x}=\frac{k_3}{k_4}b(1-e^{-k_4t}), \qquad (3)$$

therefore $$b=\frac{x+K_m\ln\dfrac{a}{a-x}}{\dfrac{k_3}{k_4}(1-e^{-k_4t})}.$$

If t is large, this becomes

$$b=\frac{k_4}{k_3}\left[x+K_m\ln\left(\frac{a}{a-x}\right)\right].$$

Now, if x is small compared with a,

$$b=\frac{k_4}{k_3}x,$$

i.e. $$b=Kx \text{ (where } K=k_4/k_3),$$

i.e. the amount of substrate finally destroyed is proportional to the initial concentration of enzyme. Since K_m is also small, x can become 0·5 to 0·6 a before there is appreciable divergence from proportionality (Fig. 2).

This analysis confirms that the method described has a satisfactory theoretical basis, although the reactions with respect to substrate decomposition are not of the first order. However, in order to convert the units of this method into those of Kat.f. it still remains necessary to determine the first-order-reaction velocity constant (substrate decomposition) for each sample.

Fig. 3 (curve *B*) suggested that provided all the substrate is destroyed at the end of the reaction then its decomposition might be of the first order. Fig. 4 confirms this and shows that the initial-reaction velocity is proportional to the substrate decomposition when this is below 0·014 M, i.e. under these conditions the reaction was of the first order.

It is therefore possible from the initial slope of Fig. 4 to calculate the first-order velocity constant for this sample since $V=k_1S$, where V is the reaction velocity, S is the substrate concentration and k_1 is the first-order-reaction velocity constant.

Euler & Josephson (1927), in order to determine Kat.f., used a reaction-velocity constant k which was a function of common logarithms ($\log_{10}$), whereas in the above formula k_1 is a function of natural logarithms (ln).

Therefore it is necessary to substitute k for k_1 in the above formula, i.e.

$$V=2{\cdot}3kS.$$

In order that k of this formula should be equivalent to k of Euler & Josephson's formula $[k=1/t\{\log_{10}(a/a-x)\}]$, where a is the initial substrate concentration and x the substrate decomposed after t min., it was found that S was not the initial substrate concentration but the mean of the initial concentration and of the concentration at the time of taking the reading. V and S can now be obtained by taking any point on the linear first-order portion of Fig. 4, so that k can be determined.

For example, take the point: 8 µl. of O_2/min.; 0·01 M-H_2O_2 added substrate. Since 3 ml. of reaction mixture is used, then 8/3 µl. of O_2 is evolved/min./ml. of reaction mixture, i.e. $V=2{\cdot}67$, and the initial substrate concentration is 0·01/3 M-H_2O_2, i.e. 37·3 µl. of available O_2/ml., and at the time of taking the reading the substrate concentration has become (37·3–8) µl. of available O_2/ml. Therefore $S=\frac{1}{2}[37{\cdot}3+(37{\cdot}3-8)]=33{\cdot}3$ µl. of available O_2/ml. Therefore

$2{\cdot}67=2{\cdot}3k\,.\,33{\cdot}3$, by substituting for V and S,

therefore $$k=0{\cdot}035 \text{ min.}^{-1}$$

Euler & Josephson's formula for obtaining the Kat.f. value for this method becomes

$$\text{Kat.f.}=k/(\text{g. of protein}\times 50/3)$$

since 3 ml. of reaction mixture was used instead of 50 ml. With this sample of blood, the ratio of haemoglobin to total blood protein was 14·6:17·4, so that a dilution of 5 µg. of haemoglobin/ml. meant that there was 11·9 µg. of total blood protein present in the reaction mixture. With these data, it was found that this blood sample had an activity of

Table 1. *Determination of the factor for converting the units of activity of whole blood, obtained by the method described, into Kat.f. units of washed red-blood cells*

k is calculated by applying the formula $V = 2{\cdot}3kS$ to the results obtained after adding 1 ml. of 0·01 M-H_2O_2 solution to 2 ml. of buffer solution containing 10 μg. of haemoglobin, the reaction being stopped after 3 min. with 2 ml. of 2 N-H_2SO_4 and the amount of substrate destroyed being measured (see text for detailed example.) The reactions are of the first order under these conditions (Fig. 4). The determination of Kat.f. for washed red cells is described in the text. The mean of the results in the last column is the factor for converting the units obtained by this method, described for whole blood, into Kat.f. units for washed red cells. This factor is 1·37.

Sample	Units of catalase activity: Whole blood	Units of catalase activity: Washed red cells	k (min.$^{-1}$)	Kat.f. (washed red cells)	Kat.f. (washed red cells) / Units of activity (whole blood)
1	160	160	0·035	210	1·31
2	156	159	0·037	222	1·42
3	188	180	0·043	258	1·37
4	170	171	0·040	240	1·41
5	166	165	0·038	228	1·37
6	201	200	0·046	276	1·37
7	144	140	0·032	192	1·34
8	183	186	0·042	252	1·38
9	191	196	0·044	264	1·38

176 Kat.f. units, a figure agreeing well with that of 166 found by Herbert & Pinsent (1948) for their sample.

Thus if the whole-blood protein is estimated it is possible to determine Kat.f. values for whole blood by this method. It was found, however, that with eight different blood samples the activities obtained by this method were the same whether whole blood was used or whether the red cells of these samples were washed free of plasma with physiological saline (0·89% NaCl) before assay (Table 1). It was also found that washed red cells contained less than 1% of protein which was not haemoglobin. It is therefore possible to dispense with whole-blood protein determinations and to convert the units of this method obtained for whole blood into Kat.f. values for washed red-blood cells, as follows:

$$\text{Kat.f.}_{\text{(washed red cells)}} = k_{\text{(whole blood)}}/(10 \times 10^{-6} \times 50/3).$$

For sample 1 (Table 1),

$$\text{Kat.f.}_{\text{(washed cells)}} = 0{\cdot}035/(10 \times 10^{-6} \times 50/3) = 210.$$

Table 1 shows that the factor for converting the units obtained for whole blood with this method into Kat.f. values for washed red cells is 1·37.

Effect of protein on measurement of catalase. Two experiments were devised to refute the possibility that the protein content of the whole-blood lysate protected the catalase against the destructive effect of hydrogen peroxide. First, a series of readings was made with identical blood samples containing 10 μg. of haemoglobin/ml. and to which had been added 1 ml. of a solution of crystalline bovine albumin so that the final quantity of 2 ml. of enzyme solution contained 0, 10, 20 and 30 μg. of albumin. The results obtained are shown in Table 2.

Table 2. *Effect of added bovine crystalline albumin on catalase activity*

Enzyme sample in 2 ml. of buffer	Activity (units)
10 μg. of haemoglobin	195
10 μg. of haemoglobin + 10 μg. of albumin	189
10 μg. of haemoglobin + 20 μg. of albumin	187
10 μg. of haemoglobin + 30 μg. of albumin	191
30 μg. of albumin	Nil

Secondly, a nearly pure sample of human red cell, haemoglobin-free catalase (Kat.f. = 23 000) was prepared by the method of Herbert & Pinsent (1948), and this was suitably diluted to give a concentration of about two-thirds of that usually found with blood samples by this method. Three different samples of blood were obtained and their activities determined. These were then diluted to 10 μg. of haemoglobin/ml. and to 1 ml. of each sample was added 1 ml. of the catalase solution. These results are shown in Table 3.

It follows that no protective action by the protein was found and furthermore that complete recovery of added catalase was obtained.

Catalase content of blood from normal and diseased subjects. This method was applied to blood samples collected from normal people, and from patients of all ages, including infants, whose blood was being investigated during routine clinical laboratory work for a wide variety of conditions, including pernicious anaemia, iron-deficiency anaemia and varieties of anaemia of secondary origin.

It is unfortunate that during the time these investigations were being conducted, no cases of anaemia associated with porphyria, or other unusual examples of altered haemoglobin metabolism, were encountered since it is in these sorts of

Table 3. *Effect of adding catalase to blood samples*

Enzyme sample	Activity (units)	Added activity (units)
Diluted catalase in 1 ml. of buffer	66	—
10 μg. of haemoglobin (sample 1) in 2 ml. of buffer	141	—
10 μg. of haemoglobin (sample 1) in 1 ml. of buffer + 1 ml. of diluted catalase	205	64
10 μg. of haemoglobin (sample 2) in 2 ml. of buffer	165	—
10 μg. of haemoglobin (sample 2) in 1 ml. of buffer + 1 ml. of diluted catalase	226	61
10 μg. of haemoglobin (sample 3) in 2 ml. of buffer	196	—
10 μg. of haemoglobin (sample 3) in 1 ml. of buffer + 1 ml. of diluted catalase	259	63

Table 4. *Results obtained with* 145 *subjects*

Figures represent units of activity; one observation was made per subject. 94% of the normal subjects and 90% of the ill subjects fell within their respective mean $\pm$S.D. limits.

	No. of subjects	Range	Mean	Standard deviation
Normal subjects	47	75–216	175	38
Ill subjects	98	106–276	184	34

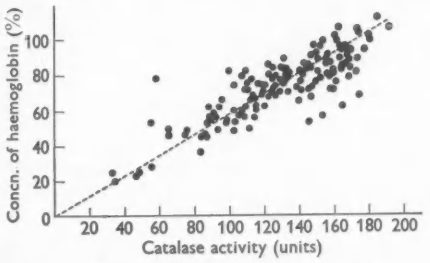

Fig. 5. A scatter diagram constructed from the results obtained during the investigation. Here the units of activity are expressed per constant volume of blood (2 ml. of 1:29 600 dilution) irrespective of the haemoglobin concentration of each sample. A concentration of 14·8 g. of haemoglobin/100 ml. of whole blood was taken as 100%. (2 ml. of 1:29 600 dilutions of 100% haemoglobin blood sample contains 10 μg. of haemoglobins.)

conditions that abnormal results might be found. The results are summarized in Table 4. No correlation was found between the catalase activity and sex or age of the persons examined.

DISCUSSION

In the method described, all the results are expressed as arbitrary units of activity relative to a constant quantity of haemoglobin and irrespective of the actual haemoglobin content of the whole-blood samples. In order to compare the activity of different blood samples, some quantity had to be common to all. Apart from the constant quantity of haemoglobin, other reference points could have been either a constant blood dilution, as used in most biochemical methods, or a constant number of red-blood cells as used by Richardson, Huddleson & Bethea (1953).

The results obtained and summarized in Table 4 show a narrow distribution, suggesting a fairly close and constant relationship between haemoglobin and catalase which is not disturbed when there is anaemia of any of the usual types, no matter how severe its degree. In order to verify this impression a scatter diagram (Fig. 5) was constructed. In this figure, the abscissa is the catalase activity of each sample expressed as units of activity/2 ml. of a 1:29 600 dilution of whole blood. Since the haemoglobin concentration of each sample was known, and since within the limits of the dilutions used in this method the units of activity of any sample was directly proportional to the haemoglobin content of that sample (see Fig. 2), it was a simple matter to derive the figures plotted from the results obtained and summarized in Table 4.

This graph shows the close linear relationship that exists between catalase activity and the haemoglobin concentration of human blood. This linear relationship suggested that the method used was in fact measuring haemoglobin and not catalase. However, Perlmann & Lipmann (1947) were unable to detect any catalase activity with crystalline haemoglobin, and a report has been published of a family in which several members showed no catalase activity in their blood despite normal haemoglobin concentrations (Takahara, 1952). Furthermore, complete recovery of haemoglobin-free catalase added to the blood samples was obtained by this method (see Table 3).

The use of a constant haemoglobin content, rather than a constant blood dilution, to compare the activity of different samples is supported by the findings shown in Fig. 5. It also justified the decision not to express the activity as a relationship of a fixed number of red cells, since their haemoglobin concentration varies widely in the different types of anaemia. Richardson, Huddleson, Bethea

& Trustdorf (1953) found that the 'index of catalase activity', referred to a constant number of red cells of cattle blood, increased steadily with age of the cattle, whereas the activity expressed/ml. of blood did not change. They also found that the red-cell count decreased with age. Their findings further support the decision to refer the activities of different blood samples to a constant haemoglobin amount, rather than to a constant number of red cells.

It would appear that these two haematin-containing compounds, besides being produced at the same site and contained in the same cell, react similarly to stresses placed upon the bone marrow and always maintain a constant proportion to each other. This similarity of response to marrow disturbance produced by various pathological processes would suggest that catalase and haemoglobin are manufactured in the marrow along a common pathway until the haematin moiety is completed, and that they then follow a different metabolic route for the addition of the globin portion to complete the molecule. Nevertheless, the two final routes must be governed, or govern each other, in some way so that the constant quantitative relationship is maintained.

SUMMARY

1. A simple titrimetric method, giving reproducible results, is described for estimating the catalase activity of blood. The results are expressed in arbitrary units which can readily be converted into the conventional Kat.f. units.

2. The principle of this method depends upon the destructive effect which hydrogen peroxide has upon the enzyme, and the quantities are arranged so that the catalase is destroyed before all the substrate has been decomposed. The reaction may therefore be allowed to proceed to completion and the time factor is no longer critical when making the actual measurements. Under these conditions, there is a linear relationship between the amount of substrate finally utilized and the quantity of enzyme present.

3. There is a constant relationship between catalase activity and haemoglobin concentration, which is not affected by anaemia, age or sex.

4. It is suggested that this relationship indicates a common metabolic pathway for the formation of haemoglobin and catalase, so far as the haematin part of the molecule is concerned.

This work was commenced with a research grant from the Governors' Discretionary Fund of the Westminster Medical School and completed with the aid of a Government Research Grant made by the Royal Society. My thanks are due to Dr H. M. T. Coles for originally suggesting the work and helping in its early stages, and to Dr E. M. Darmady for his encouragement. I am indebted to Mr H. L. J. Rolley for invaluable technical assistance and to Mr J. Durant, who has read the paper and assisted me with various chemical problems as they arose. Dr E. M. Crook kindly suggested the kinetic analysis of the reaction.

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Spectroscopic Effects of Adenosine Diphosphate upon the Respiratory Pigments of Rat-Heart-Muscle Sarcosomes

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Both the efficiency of phosphorylation and the mechanism of action of the respiratory carriers involved in this process, as found by Slater & Holton (1953, 1954), Slater (1955) and Holton (1955) with heart sarcosomes, differ from the results obtained elsewhere on heart sarcosomes (Maley & Plaut, 1953, 1954) and on liver mitochondria (Copenhaver & Lardy, 1954; Lardy & Wellman, 1952; Chance & Williams, 1955*a*). For example, Slater (1955) holds that the phosphorylation efficiency of the heart-muscle sarcosomes is lower than that of liver mitochondria although Maley & Plaut (1953) find little difference. Holton (1955), who has shown how a commercially available spectrophotometer can be used to obtain spectra representing the differences between the oxidized and reduced forms of the cytochromes of Slater's sarcosome preparation, finds that cytochrome *b* is more reduced in the presence of adenosine diphosphate (ADP) than in its absence, a result in contrast to our studies on cytochrome *b* of liver mitochondria (Chance & Williams, 1955*a*; Chance, 1955). These results have led to the proposal of a chemical basis for the difference between phosphorylation processes in heart sarcosomes and liver mitochondria (Slater, 1955).

We have now prepared heart-muscle sarcosomes according to the methods of Cleland & Slater (1953) and have recorded their phosphorylative efficiency by means of the rapid-polarographic method (Chance & Williams, 1955*b*); simultaneously the mechanism of the phosphorylation process has been recorded by means of the sensitive spectrophotometric techniques used in the study of rat-liver mitochondria. Both the phosphorylation efficiency and the mechanism of the process are found to be similar to those of liver mitochondria.

Heart-muscle sarcosomes, when studied according to our methods, meet the sensitive criterion of respiratory control as well as do liver mitochondria. The respiratory-control ratio is here defined as the extent to which respiration is slowed after phosphorylation of a given amount of ADP and is given by the respiratory rate with ADP divided by the respiratory rate after added ADP has been depleted. This is a more critical test than that afforded by a measurement of the acceleration of respiration upon addition of ADP. The sarcosomes used in this paper show respiratory control ratios of about 10 measured according to this criterion. The heart-muscle sarcosomes retain high phosphorylative activity and respiratory-control ratios long enough for study by these methods.

Spectroscopic data obtained on the oxidative-phosphorylation systems of whole yeast and ascites cells, and of intact frog and turtle muscles, are in agreement with the results presented here on heart-muscle sarcosomes, and it is probable that the sarcosome preparations studied here exhibit the biochemical properties of the intact system.

EXPERIMENTAL

Preparations

The rat-heart sarcosomes were prepared in accordance with the procedure of Cleland & Slater (1953) and a personal communication from Dr E. C. Slater in 1954. The α-oxoglutarate was recrystallized, and its low succinate content can be inferred from an enzymic assay in this paper (Fig. 9*A*). The ADP was obtained freshly from the Sigma Chemical Co. (the manufacturer's chromatographic assay of this

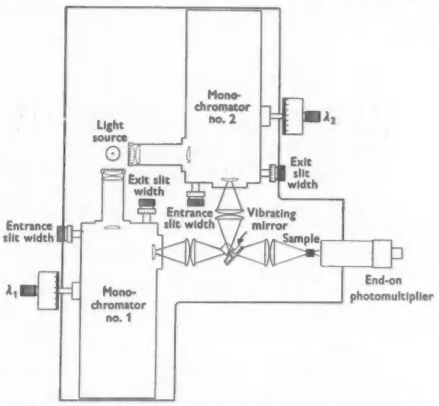

Fig. 1. Block diagram of optical components of the double-beam spectrophotometric method. A tungsten-light source illuminates two grating monochromators and the emergent beams fall upon a vibrating mirror mounted on top of a Brown 'converter' (see Fig. 2). The image of the grating is focused on the sample, which is placed close to the photomultiplier (MD 34).

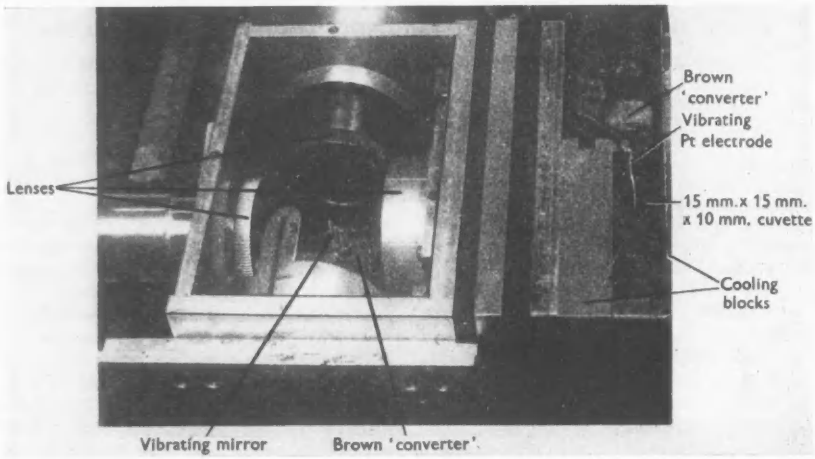

Fig. 2. Double-beam spectrophotometric method. Photograph of sample holder and vibrating platinum electrode, together with light-chopping assembly. The photocell is about 1 cm. beyond the cuvette.

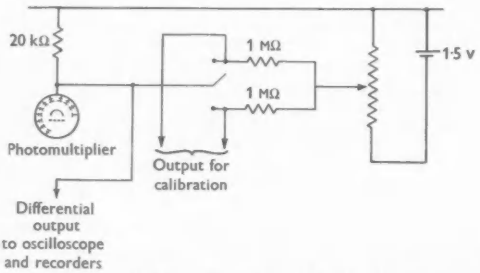

Fig. 3. Electrical connexions for calibrating the output of the double-beam spectrophotometric method. Switch contacts attached to the light-chopper are used to compare the output of the photomultiplier with an adjustable voltage. When the adjustable voltage is equal to the amplitude of the light pulses, the output of the photomultiplier is measured. The amplifier circuits can then be calibrated in terms of their response to a small increment of the adjustable voltage.

sample shows over 98% of ADP) and was assayed spectrophotometrically after each experiment. The reactions were usually carried out in a sucrose–ethylenediaminetetraacetic acid medium, but the salt medium used for liver mitochondria gave satisfactory results for brief experiments. The temperature of all these studies was 26°. The optical path length was 1 cm. except in the experiment of Fig. 5*C*.

Methods

Double-beam spectroscopic methods. All the kinetic data of this paper were recorded with the double-beam spectrophotometer (Chance, 1951, 1954*b*). As indicated in the diagram (Fig. 1), two Bausch and Lomb grating monochromators are illuminated from a photocell-stabilized tungsten lamp (Chance, 1940). The emergent monochromatic light beams are focused upon a mirror vibrating at 60 cyc./sec. that flashes the two beams alternately upon the cuvette, upon which the rectangular images of the two gratings are focused. The cuvette is held between temperature-controlled plates (Fig. 2). The photocell is of the 'end-on' type and is placed close to the 15 mm. × 15 mm. cuvette. A platinum electrode vibrating at 60 cyc./sec. is placed in the cuvette (Fig. 2), and since its vibrations are synchronous with those of the light flashes, no optical disturbance is caused. In operation, a pair of wavelengths is selected (for example, 430 and 410 mμ for cytochrome *b*) and the monochromator slits are set to an appropriate width, about 1–2 mμ (3·3 mμ/mm. dispersion). By observation of the alternating component of the photomultiplier output, the slits are adjusted so that equal photocurrents flow during the light flashes. The alternating component then indicates the difference of light absorption at the two wavelengths and is unresponsive to any non-specific absorption. The average output voltage of the photomultiplier is measured by a 'calibrate' switch which allows comparison to be made between the photomultiplier output and an adjustable voltage through contacts on the vibrating mirror (Fig. 3). The alternating component of the photomultiplier is at a minimum when the two voltages are equal. The voltage sensitivity of the amplifying circuits is measured by their response to an incremental change of the calibrating voltage. Thus the recorders are calibrated directly in terms of change of percentage transmission. This is readily converted into change of extinction by the factor 0·434 for the small changes of transmission. The fluctuations in the output of the double-beam spectrophotometer are negligible in these studies.

Split-beam spectroscopic method. For a rapid wavelength scan of the differences in extinctions between two samples of mitochondria which are in different states, such as aerobic and anaerobic, a split-beam method is used. Here one beam of light is swept across the two samples 60 times/sec. and the difference of extinction is read from the alternating-current output of a photomultiplier. The phototube response to light through the reference cuvette is maintained constant by a feed-back circuit which controls the potential applied to the dynodes. This instrument is used also for recording spectra at liquid-air temperatures. Spectral widths of less than 1 mμ are used, even with turbid materials.

Polarographic method for utilization of oxygen. The platinum-electrode circuit is calibrated in reaction medium saturated with air and in the medium made anaerobic by the respiration of the sarcosomes. Independent tests show the electrode current to respond linearly between these calibration points with an error of less than 10% of full scale (Chance & Williams, 1955*b*). The frequency of electrode calibrations was adequate to eliminate errors due to drifts in sensitivity.

Assays of preparations. The cytochrome a_3 content of each preparation was assayed spectrophotometrically, and the concentration of enzyme (μM) used in each experiment is given in each figure. Protein determinations gave 7×10^{-10} moles of cytochrome a_3/mg. of sarcosomal protein. Since a volume of 2·4 ml. is used in double-beam experiments an estimate of the protein concentration may also be obtained (2·4 ml. of μM-cytochrome $a_3 \equiv 3{\cdot}4$ mg. of protein). In Table 2 are summarized the properties of one preparation.

RESULTS

Respiration and phosphorylation

A typical experimental result of a test of these heart-muscle-sarcosome preparations for respiratory and phosphorylative activities is given in Fig. 4. Their properties are comparable with those of mitochondria prepared from liver, a test of which is reproduced for comparison on the right side of the figure. From the left edge of Fig. 4*A*, it is seen that the addition of sarcosomes causes no appreciable change of oxygen tension since this sarcosome suspension is already saturated with air owing to lack of substrate.

On the other hand, addition of the liver mitochondria to the reaction medium causes an immediate drop of oxygen tension due to a dilution of the oxygen by the anaerobic mitochondrial suspension. The suspension of liver mitochondria is anaerobic because of endogenous substrate and thereby differs from the sarcosome suspension. The absence of endogenous substrate in the heart sarcosomes is verified in Fig. 4*A* by the fact that addition of ADP to the sarcosome preparation causes negligible respiration. With liver mitochondria, utilization of about 30 μM-oxygen/l. without added substrate would have been expected under these conditions (Chance & Williams, 1955*b*, Fig. 4*B*). With the sarcosomes, respiration begins some seconds after α-oxoglutarate has been added. Respiration continues until the ADP previously added is exhausted. Then the respiration rate decreases by a factor in excess of seven. This respiratory-control ratio of seven rises to over ten for subsequent additions of ADP. The high affinity of the sarcosomal preparation for ADP is verified by the sharp break in the respiratory rate when the ADP is depleted. Less successful sarcosome

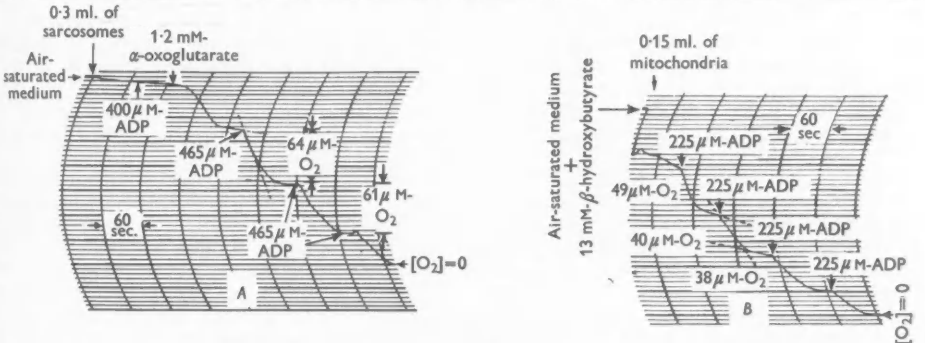

Fig. 4. *A.* A typical recording of the respiratory and phosphorylating activities of the heart-muscle sarcosomes. The vibrating platinum electrode illustrated by Fig. 2 is used in these studies in order that the stirring artifact be minimal and that its response to changes of respiratory rate be rapid. The respiratory control ratios are computed directly from this record in terms of the slopes of the traces with and without ADP, and the phosphorylation efficiency is also computed directly from the ratios of the added ADP to the increment of oxygen taken up at the rapid rate. The values of P:O are 3·7 and 3·8, the respiratory activity $(K_4)_c = 130$, 0·5 μM-cytochrome a_3 (Expt. 615). *B.* Respiratory and phosphorylative activities of rat-liver mitochondria. Addition of the mitochondria causes a rapid decrease of oxygen concentration owing to endogenous substrate in the liver mitochondria. Also the initial P:O value is smaller for the first addition of ADP than in the last two additions in which the mitochondria are operating on β-hydroxybutyrate [see Chance & Williams (1955*b*) for full details].

preparations fail to show the clear-cut 'shut-off' of respiration demonstrated by the trace of Fig. 4*A*.

A second addition of ADP speeds the respiration and then the P:O value can be determined by the quotient of the moles of ADP added and the atoms of oxygen taken up during the period of accelerated respiration. For this particular experiment, the value of 3·7 is obtained, in comparison with Maley & Plaut (1953) who obtained a value of 3·3, and these values are definitely in excess of those published by Slater (1955). The liver mitochondria, with β-hydroxybutyrate as substrate, gives a P:O value of 2·8. Successive additions of ADP verify the value of the P:O ratio in both cases and give an idea of the consistency of the method. Since the same technique was used in both cases and the ADP was assayed in the same manner (see above), it is thought that these records give an exact comparison of the phosphorylative activities of the two types of preparations. The average of the determinations on seven sarcosome preparations is 3·5 (29 individual assays).

A rather interesting effect is observed when β-hydroxybutyrate is used to initiate respiration and α-oxoglutarate is added so that respiration occurs in the presence of both substrates. In a particular experiment it was found that the P:O value did not rise, but fell by 10% upon addition of α-oxoglutarate. The nature of this effect is not understood, but a possible explanation is that the β-hydroxybutyrate dehydrogenase still dominated the reduction of diphosphopyridine nucleotide (DPN) in spite of the presence of α-oxoglutarate. This fact, coupled with the often-observed induction period in the initiation of respiration upon addition of α-oxoglutarate, supports the hypothesis that some intermediate may be formed before α-oxoglutarate can initiate respiration by reduction of DPN in the intact sarcosome.

Spectroscopic studies

Oxidized–reduced states. A split-beam-recording spectrophotometer is used to plot the spectrum representing the differences of absorption between the anaerobic (state 5) and aerobic (state 3) sarcosomes (states 3–5), and typical results are plotted in Fig. 5*A*. In state 3, the sarcosomes are supplied with substrate, phosphate, phosphate acceptor and oxygen. In state 5, they are anaerobic. The absorption bands of the reduced forms of the cytochromes, a_3, a, b and $c+c_1$, are clearly distinguished and are qualitatively similar to those already reported for yeast cells, liver mitochondria and for non-phosphorylating heart-muscle preparations. These results clearly show the α-band of cytochrome b at 562 mμ as a shoulder on the absorption band of cytochrome $c+c_1$ and, in general, give a clearer spectrum in the visible region of the spectrum than could be obtained with the slit width of roughly 20 mμ used in the corresponding study reported by Holton (1955). The trough due to the reduction of oxidized flavoprotein clearly shows at 465 mμ. In the ultraviolet region of the spectrum there is a marked difference between these results and those of Chance & Williams (1955*c*) on liver mitochondria; no distinctive 340 mμ band is recorded and the absorption gradually rises to a peak, as shown by Fig. 5*B*, in the region of 320 mμ as has recently been found by Holton (1956). This result is in considerable contrast with that obtained on adding ADP, for in this case DPN is clearly involved and has an absorption maximum at 340 mμ (Fig. 8). If the absorption band at 320 mμ is due to DPN, it is apparently not involved in the oxidative phosphorylation pathway responsive to ADP addition but may be due to a bound coenzyme in the α-oxoglutarate dehydrogenase system. This absorption band is so large relative to that of the α-bands of the cytochromes that it need not be attributed to their δ-bands (unpublished data on Keilin & Hartree heart-muscle preparations).

If β-hydroxybutyrate is used as a substrate, the absorption increasing towards 320 mμ is not as great (Fig. 5*A*), but it is nevertheless difficult to determine the contribution due to reduced DPN (DPNH) absorbing at 340 mμ, and the results are not nearly as clear as those obtained with liver mitochondria. Evidence for the presence of '340 mμ' DPN in the sarcosomes and for its participation in the respiratory chain is afforded by two specific reactions of the aerobic sarcosomes: (i) its reduction upon addition of β-hydroxybutyrate and (ii) its oxidation and reduction upon addition and exhaustion of ADP (see Fig. 8).

Liquid-air spectrum of respiratory components of rat-heart-muscle sarcosome. Fig. 5*C* shows the apparent absolute spectrum of the respiratory pigments of a sarcosome preparation made by the method of Estabrook (1956). This spectrum represents the difference between a glycerol–water blank and a glycerol–water suspension of the sarcosomes, both frozen at the temperature of liquid air, warmed to give a microcrystalline structure and then frozen again. Under these conditions the absorption band of cytochrome c_1 of the sarcosomes can be observed at a wavelength of 554 mμ as a shoulder on the much larger absorption band of cytochrome c, when the material has been reduced by succinate and the terminal oxidase inhibited by azide. The content of cytochrome c_1 is roughly half that of c, if it is assumed that c and c_1 have the same extinction coefficient. Reduction by dithionite causes the appearance of a broad absorption band that raises the level of the

bands of c, c_1 and b. The additional absorption caused by addition of dithionite may be attributed to pigments other than cytochromes c_1 and b. Although the sarcosomes show a rather small absorption band corresponding to cytochrome c_1, this component is reduced upon the addition of succinate and therefore may participate in electron transport.

Relative concentrations of the components of the respiratory chain. Quantitative data on the respiratory chain of the sarcosomes (see Figs. 5 and 8) are summarized in Tables 1 and 2. The differences in changes of absorption are measured at the pairs of wavelengths indicated and are then divided by the value for cytochrome a. This quotient gives the 'sequence of extinctions' which is then converted into a 'sequence of concentrations' by dividing each extinction by the molecular-extinction coefficient, and again referring the data to cytochrome a. For comparison, data on liver mitochondria are included. It is seen that the cytochromes are present in about the usual ratios. The absolute amount of cytochrome c is about 8×10^{-10} moles/mg. of protein, and this value is about four times higher than that found in the liver particles (Tables 1 and 2).

DPN associated with the oxidative-phosphorylation system is assayed by both the split-beam and double-beam methods. In split-beam recordings similar to those illustrated by Fig. 8*A*, the

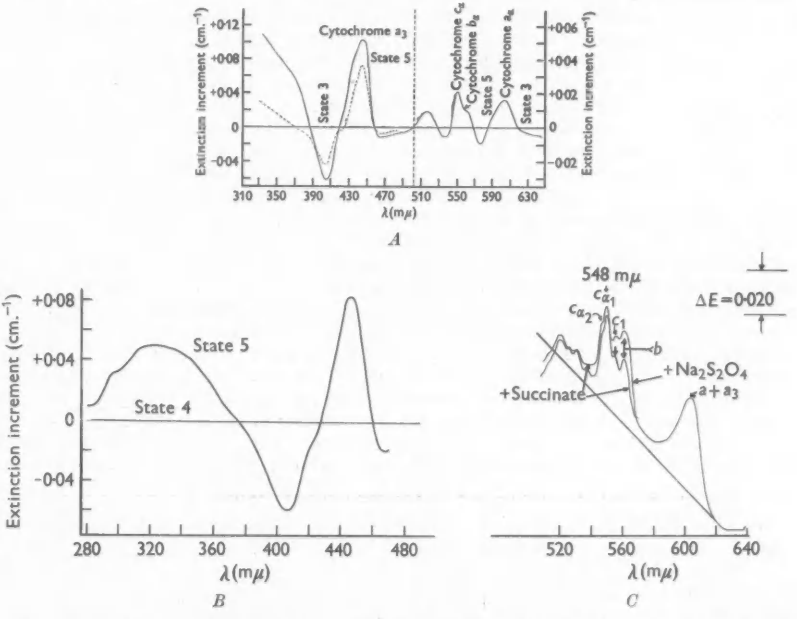

Fig. 5. Spectra representing the extinction differences between reduced and oxidized components of the respiratory chain of the sarcosomes. *A*. With the solid curves, both ADP and α-oxoglutarate are present and one suspension is allowed to become anaerobic before the other. The record shows the differences of extinction between the two samples and corresponds to the change between states 3 and 5. The broken curve is a similar record with another preparation for β-hydroxybutyrate as the substrate. Under such conditions, cytochromes b and c are incompletely oxidized (see Table 3), and DPN would be about half-oxidized in the aerobic sample. It should be noted that the ordinate scale of the right-hand portion of the figure is twice that of the left-hand portion of the figure (1·2 μM-cytochrome a_3) (Expt. 616*d*). *B*. A detailed recording of the ultraviolet-absorption difference spectrum (oxidized minus reduced) of heart-muscle sarcosomes. Contents of both cuvettes are treated with α-oxoglutarate and sufficient ADP to cause anaerobiosis in one cuvette. The spectrum record under these conditions represents the difference between steady-state oxidized (state 4) and anaerobic sarcosomes (state 5). A Bausch and Lomb monochromator fitted with a hydrogen-discharge lamp is used in recording this spectrum. The slit width is 6 mμ. The broad absorption peak is at too short a wavelength to be attributed to the DPN which is observed to be oxidized and reduced upon the addition of ADP (see Fig. 8*A*) (1·2 μM-cytochrome a_3) (Expt. 674*b*). *C*. The apparent absolute spectrum of the cytochrome components of heart-muscle sarcosomes recorded at temperatures of liquid air. Slit width is 0·6 mμ. The optical path is 1 mm. (5·4 μM-cytochrome a_3) (Expt. 614*d*).

increment of absorption at 340 mμ caused by the addition of β-hydroxybutyrate to the aerated substrate-free sarcosomes (state 2) is measured and is found to correspond to 20 % of the change measured at 445 mμ when the sarcosomes become anaerobic (Table 1). Experiments with the split-beam spectrophotometric method give, for the particular preparation used, an increment at 340 mμ, measured with respect to 374 mμ, of about 30 % of the value for cytochrome a_3. Addition of α-oxoglutarate at sufficiently low concentration to avoid spectroscopic interference gives similar results. On the basis of such experiments we reach the value for DPN content of the oxidative-phosphorylation system given in Table 1.

If sarcosomes to which substrate has been added are allowed to become anaerobic, a further increment of extinction is registered at 340 mμ. The split-beam recording of Fig. 5*B* indicates that this material has a peak at 320 mμ and not at 340 mμ. Since Fig. 8 shows that DPN that participates actively in oxidative phosphorylation absorbs at 340 mμ, the additional absorption illustrated by Fig. 5*B* is due to another component. DPN involved in phosphorylation of liver and kidney mitochondria shows an absorption maximum very close to 340 mμ. It is concluded that the DPN associated with phosphorylation in the sarcosomes is also largely reduced in state 4, for the following reasons: (i) no additional 340 mμ peak appears when sarcosomes in state 4 are allowed to become anaerobic (see Fig. 5*B*); (ii) both the liver and the heart preparations have a high respiratory-control ratio which depends upon a large reduction of DPN in state 4 (Chance, 1953); (iii) addition of ADP to liver mitochondria in state 4 causes about 50 % oxidation of DPN, and similar results are observed with sarcosomes (50 % oxidation occurs with β-hydroxybutyrate and 35 % with α-oxoglutarate as substrate).

These assays (Table 1) indicate that the respiratory chain of heart sarcosomes is relatively deficient in flavoprotein and DPN, the ratios between sarcosomal and mitochondrial flavoprotein and DPN contents being 1/3, and 1/6 to 1/15 respectively. Thus we are able to confirm our supposition that the respiratory chain of the rat-heart sarcosomes contains relatively less DPN than liver mitochondria, in contrast with Holton's conclusion (1955, 1956). Interference due to other cytochromes is minimized by the use of specific treatments which affect as few components other than DPN as possible; for example, the transition from state 2 to state 4 or Amytal treatment of sarcosomes in state 3. Light-scattering effects are minimized by using dilute suspensions, and such effects would over- not under-estimate the DPN content.

Steady states of the respiratory enzymes. The various states of the sarcosomal respiratory enzymes are illustrated by the sequence of reactions in Fig. 6, recorded in the double-beam spectrophotometer. Sarcosomes diluted in the reaction medium are, by definition, in state 1, with inorganic phosphate but without added ADP. These sarcosomes contain a little substrate, and the respiratory carriers are partly reduced in state 1.

The addition of ADP gives an abrupt upward deflexion corresponding to a decrease of extinction at 430 mμ. As later results show, this is caused by a disappearance of the absorption band of reduced cytochrome *b*, i.e. an oxidation of reduced cytochrome *b* has occurred. The sarcosomes briefly respire with the small amount of endogenous

Table 1. *Sequence of extinctions and relative concentrations of respiratory enzymes in rat-heart sarcosomes (Expts. 616d, 621d, 620c, 622a)*

Wavelength pair (mμ)	{605 / 630	562 / 575	550 / 540	465 / 510	445 / 465	340 / 374
Component	*a*	*b*	*c* (+c_1)	*fp*	a_3	DPNH*
Sequence of extinctions (cm.$^{-1}$)	1·0	1·2	1·6	0·8	6·4	1·6
Millimolar extinction coefficient (cm.$^{-1}$ mM^{-1})	16	20	19	11	91	5·8
Relative concentrations (sarcosomes)	1·0	1·0	1·3	1·1	1·1	4·3
Relative concentrations (liver mitochondria)	1·0	0·9	1·7	3·6	—	19 (40 max.)

* β-Hydroxybutyrate was added to sarcosomes in state 2 to give state 4.

Table 2. *Characteristics of rat-heart sarcosome preparation; α-oxoglutarate as substrate (Expt. 619)*

For definitions of terms see Chance (1952). TN = turnover number.

								10^{10} × Content (moles/mg. of protein)		
								Cytochromes		
Q_{O_2}	K_1	K_3	$(K_4)_{a_3}$	TN_a (sec.$^{-1}$)	TN_c (sec.$^{-1}$)	Respiratory-control ratio	P:O	a_3	$c+c_1$	DPN
75	2	40	52	21	16	16 (max.)	3·5	7	8	20

substrate. After a pause of about 10 sec. (Fig. 6*A*) the trace rises again, but more slowly, for about 1·5 min. This time it is the exhaustion of the endogenous substrate that leads to the further and complete oxidation of cytochrome *b*, which is, by definition, state 2, the substrate-free or 'starved' state. A similar sequence of reactions is recorded in Fig. 6*B*. Addition of either β-hydroxybutyrate (Fig. 6*A*) or α-oxoglutarate (Fig. 6*B*) causes an abrupt reduction of cytochrome *b* as a consequence of reduction of DPN. In Fig. 6*A*, sufficient ADP is present to show a clear-cut plateau corresponding to the active or phosphorylating state 3. In Fig. 6*B*, the steady state is not established before the added ADP is phosphorylated. This slow reaction is probably due to the build-up of intermediates in the α-oxoglutarate oxidase system, as contrasted with the rapid establishment of the steady state in the β-hydroxybutyrate oxidase system. The fact that nearly complete oxidation of cytochrome *b* is obtained in the presence of Amytal shows that this intermediate is probably not succinate. A further addition of ADP in both cases gives an oxidation of cytochrome *b* which persists until the added ADP is phosphorylated, about 1 min. The extent of oxidation caused by ADP in the presence of β-hydroxybutyrate is nearly half that obtained in state 2. The oxidation of cytochrome *b* caused by addition of ADP in the presence of α-oxoglutarate is very definite, but considerably smaller than that caused by β-hydroxybutyrate for this particular preparation.

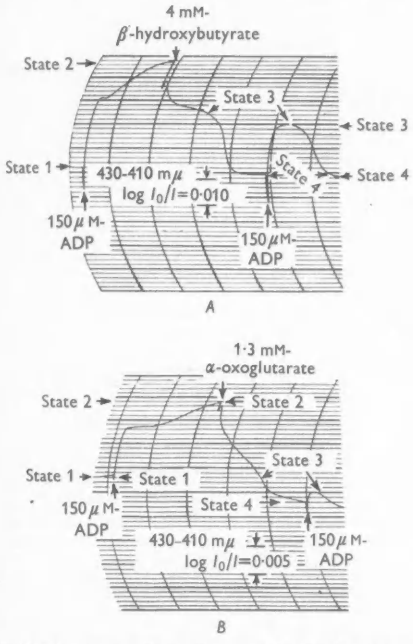

Fig. 6. The states of cytochrome *b* in the heart-muscle sarcosomes. An upward deflexion of the traces represents a decrease of extinction at 430 mμ with respect to 410 mμ and is interpreted as an oxidation of cytochrome *b*. The increment of time between each of the curved divisions is 60 sec. The sarcosomal concentration is approximately the same as that used in the record of Fig. 4. The oxidation–reduction levels corresponding to the various steady states are indicated in the diagram and are defined in the text. In *A*, the substrate is β-hydroxybutyrate (μM-cytochrome a_3), and in *B* the substrate is α-oxoglutarate (0·5 μM-cytochrome a_3) (Expt. 621*b*).

The relationship between the extent of reduction of cytochrome *b* and the steady state (state 3) on the one hand and the rate of respiration on the other is indicated by Fig. 7, and the close correlation of these two effects is clearly shown. The apparent Michaelis constant is about 0·33 mM.

In order to identify the respiratory components affected by the changes of concentration of ADP, we have used the split-beam recorder to plot the spectrum representing the differences between states 3 and 4 (see Fig. 8*A*), i.e. the absorption changes that occur when the added ADP is exhausted. The difference spectra are plotted for both α-oxoglutarate and β-hydroxybutyrate in the region of the Soret band, and for α-oxoglutarate in the visible region of the spectrum. The absorption bands of cytochrome *b* and of DPNH appear at 430 mμ and near 340 mμ respectively for both substrates. The band of DPNH is shown more clearly and accurately in Fig. 8*B*. The α-band of cytochrome *b* is observed at 562 mμ. The trough at 605 mμ is due to the disappearance of reduced

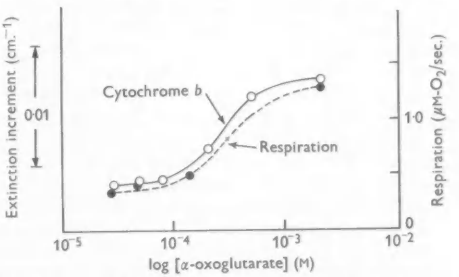

Fig. 7. A correlation between the extent of reduction of cytochrome *b* and the respiratory rate in the presence of α-oxoglutarate. The left-hand scale indicates the increment of optical density corresponding to the reduction of cytochrome *b* and the right-hand scale is the respiration rate in μM-oxygen/sec. (0·45 μM-cytochrome a_3) (Expt. 618*a*).

cytochrome *a*. The change in the steady state of cytochrome *c* is not absolutely clear from this recording, but independent tests with the double-beam spectrophotometer at 550 mμ support the existence of a trough due to the oxidation of reduced cytochrome *c*. The trough in the region of 480 mμ is due to the reduction of oxidized flavoprotein. In summary, DPNH, flavoprotein and cytochrome *b* are reduced as the ADP concentration falls to zero, whereas cytochromes *c* and *a* are oxidized, and the reverse occurs when ADP is added to state 4. A quantitative study of the changes in cytochromes *b*, $c+c_1$ and flavoprotein is given in Table 3. The percentage changes in these components are considerably more distinct than those observed with guinea-pig-liver mitochondria.

Experiments similar to those of Fig. 6 have been carried out at wavelengths appropriate for the measurement of DPNH, and the 'cycles' of oxidation and reduction of DPN are found to be closely

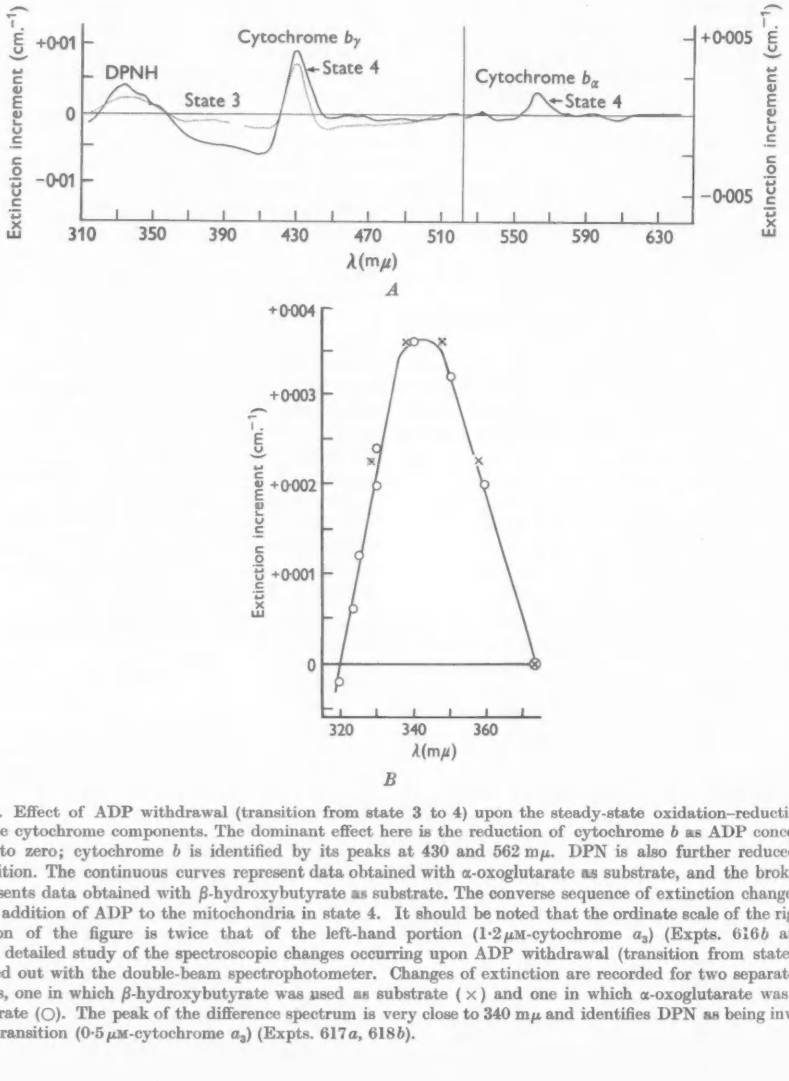

Fig. 8. *A*. Effect of ADP withdrawal (transition from state 3 to 4) upon the steady-state oxidation–reduction levels of the cytochrome components. The dominant effect here is the reduction of cytochrome *b* as ADP concentration falls to zero; cytochrome *b* is identified by its peaks at 430 and 562 mμ. DPN is also further reduced in this transition. The continuous curves represent data obtained with α-oxoglutarate as substrate, and the broken curve represents data obtained with β-hydroxybutyrate as substrate. The converse sequence of extinction changes occurs upon addition of ADP to the mitochondria in state 4. It should be noted that the ordinate scale of the right-hand portion of the figure is twice that of the left-hand portion (1·2 μM-cytochrome a_3) (Expts. 616*b* and 620). *B*. A detailed study of the spectroscopic changes occurring upon ADP withdrawal (transition from state 3 to 4) carried out with the double-beam spectrophotometer. Changes of extinction are recorded for two separate experiments, one in which β-hydroxybutyrate was used as substrate (×) and one in which α-oxoglutarate was used as substrate (○). The peak of the difference spectrum is very close to 340 mμ and identifies DPN as being involved in this transition (0·5 μM-cytochrome a_3) (Expts. 617*a*, 618*b*).

Table 3. *Effect of adenosine diphosphate upon the steady states of respiratory carriers of heart-muscle sarcosomes, α-oxoglutarate as substrate (Expt. 615c)*

State		Percentage reduction in steady state			
		Cytochrome			
No.	Characteristics	$c+c_1$ (550–540 mμ)	b (564–574 mμ)	DPN (340–374 mμ)	Flavoprotein (465–510 mμ)
1	Endogenous substrate†	5	20	—	50
2	Excess of ADP, no substrate	0	0	0	0
3	ADP, substrate present	15	36	0	35
4	Substrate present, no ADP	8	63	25	(100)
5	No oxygen	100	100	100*	—
		Cross-over point			

* Determined by treatment with antimycin-A. † When present.

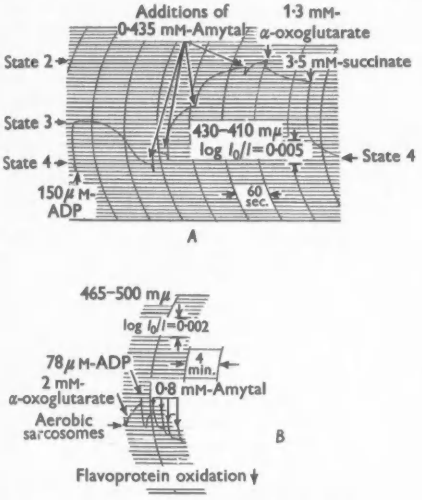

Fig. 9. *A*. Effect of Amytal upon the steady-state oxidation–reduction level of cytochrome *b*. Oxidation of cytochrome *b* corresponds to an upward deflexion of the trace of the double-beam spectrophotometer recording. Other features of the record are described in the text (0·5 μM-cytochrome a_3) (Expt. 621*a*). *B*. Effect of Amytal upon the steady-state oxidation–reduction level of flavoprotein of heart-muscle sarcosomes. Conditions are generally as in Fig. 9*A*, except that wavelengths used in recording are appropriate to flavoprotein. Note that a downward deflexion of the trace corresponds to an increase of extinction and an oxidation of flavoprotein (1·8 μM-cytochrome a_3) (Expt. 674*b*).

synchronized with those of cytochrome *b*. If these 'cycles', caused by the addition of ADP, are recorded at a variety of wavelengths, we obtain for the state 3 to state 4 transition a peak at 340 mμ (Fig. 8*B*). A similar experiment carried out with β-hydroxybutyrate gives very nearly the same curve. Both these experiments show the characteristic absorption band of DPNH at its proper wavelength, 340 mμ, to within the experimental error. In the β-hydroxybutyrate system the specificity of the enzyme for DPNH identifies the type of pyridine nucleotide, and the equality of the extinction changes in Fig. 8*B* provides a reasonable basis for the presumption that DPNH is involved at some point in the α-oxoglutarate oxidase system.

Effect of Amytal. Amytal inhibits the α-oxoglutarate oxidase system of the heart-muscle sarcosomes at concentrations that do not inhibit the succinic oxidase system. On the basis of the data of Ernster, Jalling, Löw & Lindberg (1955), this result would give independent confirmation of the results above, which show DPN to be operative in α-oxoglutarate oxidation in the sarcosomes.

Fig. 9 illustrates, in terms of the oxidation and reduction of cytochrome *b*, the inhibition of the DPNH oxidase system of the sarcosomes by Amytal and, in addition, the results show that Amytal inhibits the respiratory chain of the sarcosomes below cytochrome *b* in accordance with results obtained with liver mitochondria (Chance, 1956). Beginning at the left-hand side of the record, we observe a typical 'cycle' of oxidation and reduction of cytochrome *b* upon addition of ADP in the presence of the DPN-linked substrate, β-hydroxybutyrate. Successive additions of 0·4 mM-Amytal cause oxidation of the steady-state level of cytochrome *b* until an end-point of complete oxidation is reached and electron transport from this DPN-linked substrate is blocked. Half-maximal inhibition of respiration requires about 0·7 mM-Amytal. Electron transport from non-DPN-linked substrates, such as succinate, is not inhibited. Thus if α-oxoglutarate falls into the latter class of dehydrogenases, a large reduction of cytochrome *b* and a rapid respiration would be expected to occur upon addition of α-oxoglutarate to the material in state 2. This is, however, not the case. Only a small reduction of cytochrome *b* occurs and negligible respiration is recorded. But

addition of succinate gives a rapid respiration and a large reduction of cytochrome *b*. Judging from the 180-fold difference between the reduction rate of cytochrome *b* with α-oxoglutarate or succinate, the effect observed upon addition of α-oxoglutarate would be explained by a succinate impurity of 2%. Thus no appreciable activity of the α-oxoglutarate oxidase system can be obtained in the presence of Amytal.

In purified DPNH–cytochrome *c* reductase systems of heart muscle it has been found that Amytal apparently reacts between cytochrome *b* and flavoprotein (R. W. Estabrook, unpublished work); this is in contrast with intact liver mitochondria, where the point of attack is between DPN and flavoprotein. We have therefore determined, as illustrated by Fig. 9*B*, the point at which Amytal interacts with the respiratory chain of intact sarcosomes. Fig. 9*B* shows a spectrophotometric recording of the extinction changes between 465 and 500 mμ. Flavoprotein is reduced upon addition of α-oxoglutarate to give the quiescent state 4. Addition of ADP gives an increase of extinction corresponding to an oxidation of flavoprotein at 465 mμ. As the added ADP is exhausted, the trace returns towards its initial value. At this point a series of Amytal additions is made and the successive downward deflexion of the trace corresponds to the increased absorption of oxidized flavoprotein. Thus Amytal affects the respiratory chain of the heart-muscle sarcosomes in the same place as it does in liver mitochondria.This result suggests that a change in the pathway of electron transfer has occurred in the preparation of the reductase.

DISCUSSION

In a previous paper (Chance, 1955) it was pointed out that Slater's experimental data (Slater, 1955) on his preparations distinguish them from liver preparations in four ways: their less-marked respiratory control, their relatively low DPN content, their low P:O value, and the peculiar kinetic behaviour of cytochrome *b*. The hypothesis was advanced that muscle-sarcosome preparations so far studied by Slater were somehow damaged in the course of their preparation, especially since respiratory-control ratios obtainable in the intact muscle were large compared with any demonstrated for isolated sarcosomes. The simultaneous respiratory and spectrophotometric recordings of Fig. 10 show that in three of the four ways listed above the sarcosome preparations used in these studies behave like liver preparations. From the left-hand edge of the record, the downward deflexion of the spectrophotometric trace indicates reduction of cytochrome *b* upon addition of α-oxoglutarate. Since the ADP concentration already limits the respiratory rate, no increase of rate is observed upon addition of α-oxoglutarate in spite of the increased reduction of cytochrome *b*. Upon addition of ADP there is an abrupt oxidation of cytochrome *b* which is accurately synchronized with the increased respiratory rate. The steady state is then maintained until the ADP concentration falls to the vicinity of the 'affinity' value. Then the reduction of cytochrome *b* increases synchronously with the decrease of respiratory rate. The eightfold decrease of respiratory rate illustrates the degree of respiratory control that can be obtained with these sarcosomes. The close synchronization of the response of cytochrome *b* and the respiratory rate which is indicated by Fig. 10 is characteristic of the phosphorylation reaction studied in rat- and guinea-pig-liver mitochondria and on which a detailed theory of the mechanism of oxidative phosphorylation is based (for a summary, see Chance & Williams, 1956*b*).

The discrepancy between the P:O values of Slater and of Lardy and his co-workers is clarified by these data. Although Slater (1955) obtained the same results as Maley & Plaut (1953) on a P:α-oxoglutarate basis, he has not yet published a P:O value as large as 3; his most recent mean value is 2·86. We find a value of 3·5 for α-oxoglutarate oxidation by rat-heart-muscle sarcosomes, a value that slightly exceeds that obtained by Maley & Plaut (1953), who obtain a value of 3·3. This method gives a value of P:O for β-hydroxybutyrate oxidation by rat-liver mitochondria of 2·6, which agrees very well with the value of 2·5

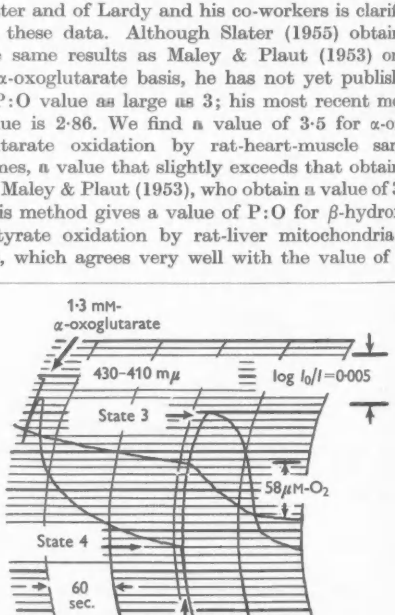

Fig. 10. A correlation of a double-beam spectrophotometric recording of cytochrome *b* with a platinum microelectrode recording of respiratory and phosphorylative activity. Other features of the record are described in the text (0·6 μM-cytochrome a_3) (Expt. 616*b*).

obtained by Copenhaver & Lardy (1954). The increment between the β-hydroxybutyrate and α-oxoglutarate values is between 0·8 and 0·9, as would be expected for the additional 'substrate-level' phosphorylation of the α-oxoglutarate oxidase system. In view of the general agreement of our results with those of the Wisconsin group, the higher values are probably correct. The difference of these values from integers may not be of as great significance as was believed in the past, since it has been shown that competitive processes in oxidative phosphorylation could lead to fractional values (Chance & Williams, 1956*b*); for example, by a competition between ADP and an adjacent member of the electron-transport chain for the inhibited 'high-energy' complex of the respiratory enzymes.

The spectrophotometric effects that we find upon addition of ADP are also consistent with the high phosphorylation efficiency. First, the oxidation of DPNH and of cytochrome *b* upon addition of ADP to the rat-heart sarcosomes is also found in liver mitochondria. Secondly, the cross-over point for the effect of ADP upon the oxidation–reduction levels of the cytochromes is located between cytochromes *b* and *c*. This response suggests that the balance of oxidase and dehydrogenase activities in the sarcosomes is similar to that of guinea-pig mitochondria, which show identical cross-over behaviour. Rat-liver mitochondria also show a cross-over point between cytochromes *b* and *c*, provided that the oxidase–dehydrogenase balance is adjusted with a low concentration of azide as has been shown by Chance & Williams (1956*a*). Thus it is reasonable to conclude that closely similar phosphorylation and electron-transfer mechanisms obtain in the sarcosome and mitochondrial systems, and that the fundamental differences in mechanism and in the nature of the respiratory chain which might have been inferred from the effects of ADP and the lower phosphorylation efficiencies of the sarcosome preparations in Slater's experiments (1955) are improbable.

The cause of the lower phosphorylation efficiency observed by Slater (1955) is difficult to determine. Making the reasonable assumption that the respiratory control is a criterion of the integrity of the sarcosomes, we find from the work of Slater & Holton (1953) that the preparation as studied by the manometric technique was definitely inferior in this respect. Although respiratory control seems not to have been considered by those workers as a factor relevant to phosphorylation, their Fig. 7 (Slater & Holton, 1953) gives data adequate for the characterization of one particular preparation; the relevant curves of their fig. 7 are 3 and 7. In 3, ADP has been added and 'by the time the first manometric reading was taken, nearly all the ADP would be phosphorylated to ATP in the absence of hexokinase'. Thus this curve corresponds to our state 4. Curve 7 corresponds to our state 3, where hexokinase is present and the ADP level is maintained constant by the enzyme system. Between the fifteenth and the twentieth minutes, the quotient of these two rates was 1·3, and between the thirtieth and the thirty-fifth minutes, the quotient is 2·1. Assuming that this preparation was typical of others that Slater and Holton used, it can be concluded that the preparations showed not only low P:O ratios, but also low respiratory-control ratios. Whereas it is probable that our rapid methods for the assay of phosphorylative and respiratory activities are of considerable advantage in the study of sarcosome preparations, the manometric method cannot be completely at fault in view of the high phosphorylative activities obtained by Maley & Plaut (1953), and some other aspect of the preparative and experimental procedures must still be considered to explain the results of Slater and Holton.

The studies of Holton (1955), who concluded that cytochrome *b* is more reduced in the presence of ADP than in its absence, differ from these in two ways: (i) his spectrophotometer was sensitive to artifacts caused by swelling of the sarcosomes; (ii) his measurements of respiratory and phosphorylative activity were not made simultaneously. It seems possible either (i) that he observed an artifact, since DNP and ADP caused opposite spectroscopic effects, or (ii) that the sarcosomes used by him were damaged either before or during his experiment, and therein differed from the sarcosomes used in this study. With regard to point (i), it does not seem to be clearly understood that the commercially available spectrophotometer used by Holton will respond equally well to non-specific extinction changes caused by swelling of the sarcosomes and to specific spectroscopic changes. For example, Slater (1955) states: 'Holton's methods were similar to those used by Chance.' We have found it necessary to use the 'double-beam' spectrophotometer for studies of mitochondrial and sarcosomal preparations. The commercially available type of spectrophotometer such as Holton used is satisfactory for studies of non-phosphorylating particulate preparations and for some types of intact cells. Holton has clearly demonstrated, however, that his technique is suitable for measuring the changes from completely oxidized to completely reduced respiratory pigments of sarcosomes.

The relative amounts of the cytochromes of the rat-heart sarcosomes are closely similar to those of other respiratory systems but considerably less flavoprotein and DPN are present. Three possible explanations for the differences between the

amounts of DPN active in phosphorylation in liver mitochondria and sarcosomes are: (i) that DPN is dissolved out of the sarcosomes during the process of preparation; (ii) that only relatively small amounts are present in the intact sarcosomes; (iii) that only a part of the total DPN is active in phosphorylation. Studies of intact frog sartorius muscle show that the increase of intracellular ADP concentration caused by contractions oxidizes about 10 moles of DPNH/mole of cytochrome a_3 (Connelly & Chance, 1954; Chance, 1953). This corresponds to the experiments of Fig. 8*A* and *B* on the rat-heart sarcosomes, in which the quotients are between 0·4 and 1. If these sarcosomes are comparable, alternative (i) is favoured. A definitive test requires direct spectrophotometric observations of DPN in rat-heart muscle.

Assay of the DPN content of the sarcosomes by the extinction changes that occur in the transition from aerobic (state 4) to anaerobic conditions may be misleading, because no distinct peak is observed at 340 mμ but, instead, one is observed at 320 mμ (Fig. 5*B*). Such material may be bound DPN (Chance, 1952; Holton, 1955, 1956), but it is not responsive to the very specific effects of ADP upon respiration and phosphorylation (cf. Figs. 5 and 8*B*). Chemical assays of the total amount of DPN in sarcosomes have the same drawback, since they do not differentiate between 'bound' and 'free' DPN. Holton (1955) finds 6×10^{-9} μmoles of DPN/mg. of sarcosomal protein. While this is a high content of DPN on a weight basis, it is low relative to the high content of cytochrome *c* in the sarcosomes (see Table 2). Table 2 further shows only 2×10^{-9} μmoles of DPN/mg. of protein. Assuming that the two types of DPN assay are comparable, it is suggested that only one-third of the total DPN participates in the phosphorylation process. Thus of the three alternatives above, it is suggested that both 1 and 3 apply to the heart-muscle sarcosomes. A relatively low concentration of DPN itself need not impair the function of the respiratory chain: the respiratory chain has a very high affinity for DPNH in the intact cell (Chance, 1954*a*) and in particles isolated from ox heart (Estabrook & Mackler, 1956). The relatively high turnover number of cytochrome *a* of the sarcosomes (21/sec.) compared with that of liver mitochondria (9/sec.) further supports this view.

Holton (1955, 1956) has not so far reported spectroscopic evidence for DPN absorbing at 340 mμ in heart sarcosomes. Thus his conclusion that DPN participates in the α-oxoglutarate oxidase system is uncertain. We are now able to put forward the following evidence for the participation of DPN in the respiration and phosphorylation in the heart sarcosomes.

(i) DPN absorbing at 340 mμ has been shown to participate in the respiratory chain of rat-heart sarcosomes on the basis of direct reduction upon the addition of the specific DPN-linked substrate, β-hydroxybutyrate, and the simultaneous initiation of respiration.

(ii) DPN absorbing at 340 mμ is oxidized and reduced in the respective presence and absence of ADP to the same extent with α-oxoglutarate as with β-hydroxybutyrate.

(iii) The sensitivities of the β-hydroxybutyrate and the α-oxoglutarate oxidase systems of the sarcosomes to Amytal are about equal.

These data show that our original results on the spectroscopic changes caused by an increase of ADP concentration are not restricted to rat- and guinea-pig-liver mitochondria but apply also to rat-heart sarcosomes. Elsewhere we report similar effects in guinea-pig-kidney mitochondria and related effects in fly sarcosomes. In intact yeast and ascites-tumour cells, the fundamental phenomenon can also be observed when the intracellular ADP level is increased by the activation of hexokinase by addition of glucose (Chance, Connelly & Hess, 1955; Chance & Hess, 1956). In our preliminary results on the spectroscopic changes after a muscle twitch, these changes have now been clearly shown to involve cytochrome *b* of frog (Weber, 1957) or turtle muscle (Jöbsis & Chance, 1957) in a manner analogous to that observed in the isolated sarcosomes. In summary, a generality of the phenomenon of readily measurable spectroscopic changes caused by changes of phosphate-acceptor concentration is fairly well established.

SUMMARY

1. The results of Maley & Plaut (1953) on phosphorylative activities of rat-heart sarcosomes are confirmed and their P:O values are slightly exceeded: the average value of P:O ratio for α-oxoglutarate oxidation found here is 3·5.

2. A high degree of respiratory control has been demonstrated in these sarcosomal preparations and values for the ratio of more than 10 have often been obtained.

3. The relative amounts of the cytochrome components of the respiratory chain of the rat-heart sarcosomes are similar to those of rat-liver mitochondria, whereas the content of pyridine nucleotide relative to cytochrome is between one-sixth and one-thirteenth of that of the respiratory chain of liver mitochondria.

4. Spectroscopic studies show that the initiation of oxidative phosphorylation by addition of adenosine diphosphate causes an oxidation of reduced pyridine nucleotide (absorbing at 340 mμ) and of cytochrome *b*. The latter result is not in agreement with that of Holton (1955) on cyto-

chrome *b*, and two explanations for his result are considered.

5. The cross-over point for the effect of addition of adenosine diphosphate upon the steady-state oxidation–reduction level of the cytochromes lies between *b* and *c*. This result is consistent with observations on mitochondrial preparations and indicates the similarity of the electron-transfer and phosphorylation mechanisms in heart sarcosomes and in liver mitochondria.

6. The observations suggest that the response of the oxidation–reduction levels of the components of the respiratory chain to increases and decreases of concentration of adenosine diphosphate is a basic characteristic of the electron-transfer and phosphorylation mechanism and is to be found in sarcosomes and mitochondria of many cell types.

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Light-Scattering and Absorption Effects Caused by Addition of Adenosine Diphosphate to Rat-Heart-Muscle Sarcosomes

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Holton (1955) has applied the Uvispek spectrophotometer to the measurement of light-absorption changes in suspensions of rat-heart-muscle sarcosomes. Although his recordings of the extinction changes caused by complete reduction of the cytochrome components are in agreement with those obtained in this Laboratory, the spectroscopic changes caused by the addition of adenosine diphosphate (ADP) to the aerobic sarcosome suspension differ sharply. Possible causes for this discrepancy have been discussed by Slater (1955 and by Chance & Baltscheffsky (1958). The experiment described in this paper appears to explain the discrepancy completely.

EXPERIMENTAL

Materials. The reagents used here were similar to those used by Chance & Baltscheffsky (1958). The rat-heart-sarcosome preparation was based upon the method of Cleland & Slater (1953*a*).

Methods. In the previous paper, a spectroscopic method especially designed to be insensitive to light-scattering

changes was used. This consists of a double-beam spectrophotometer in which the difference in extinction between two closely spaced wavelengths was recorded. This method can, in addition, be used to measure simultaneously the extinction change at either one of the wavelengths used in the double-beam method (Chance, 1951). We have therefore selected one of the two wavelengths to be suitable for measuring non-specific light-absorption effects caused by changes in light-scattering of the sarcosomal suspension (443 mμ) and the other wavelength for measuring the oxidation and reduction of cytochrome *b* (430 mμ). Since these wavelengths differ by only 13 mμ, the difference between the extinctions at these two wavelengths will be relatively insensitive to the non-specific effects. The data given in Fig. 8*A* of Chance & Baltscheffsky (1957) show that negligible specific spectroscopic changes occur at 443 mμ upon addition of ADP to the aerobic sarcosome suspension.

RESULTS AND DISCUSSION

In the top trace of Fig. 1, the phosphorylative and respiratory activities of the sarcosomal suspension are recorded by the vibrating platinum microelectrode. Immediately after the addition of 1·2 μmoles of α-oxoglutarate to give a final concentration of 0·8 mM, the respiration rate remains slow at a value of 0·2 μM-oxygen/sec. Upon the addition of 1·2 μmoles of ADP to give a final concentration of 480 μM, the respiration accelerates 5·5-fold and remains so until the oxygen concentration has decreased by 72 μM. Thereupon the respiratory rate drops by a factor of 7. The addition of 0·02 μmole of Dicumarol [3:3′-methylenebis-(4-hydroxycoumarin)] to give a final concentration of 8 μM then causes a sixfold increase of respiration.

The double-beam spectroscopic record shows, immediately after the addition of α-oxoglutarate, an upward deflexion of the trace corresponding to an increase of extinction at 430 mμ compared with that of 443 mμ. This is attributed to the reduction of cytochrome *b*. The addition of ADP causes a downward deflexion of the trace that represents the characteristic decrease of extinction at 430 mμ. This is attributed to the oxidation of cytochrome *b*. The oxidation level of cytochrome *b* decreases somewhat while the ADP is being phosphorylated and then falls sharply while the ADP concentration falls towards zero. Addition of Dicumarol (0·02 μmole to give a final concentration of 8 μM) causes an abrupt reoxidation of cytochrome *b*, and a steady state obtains until the end of the record.

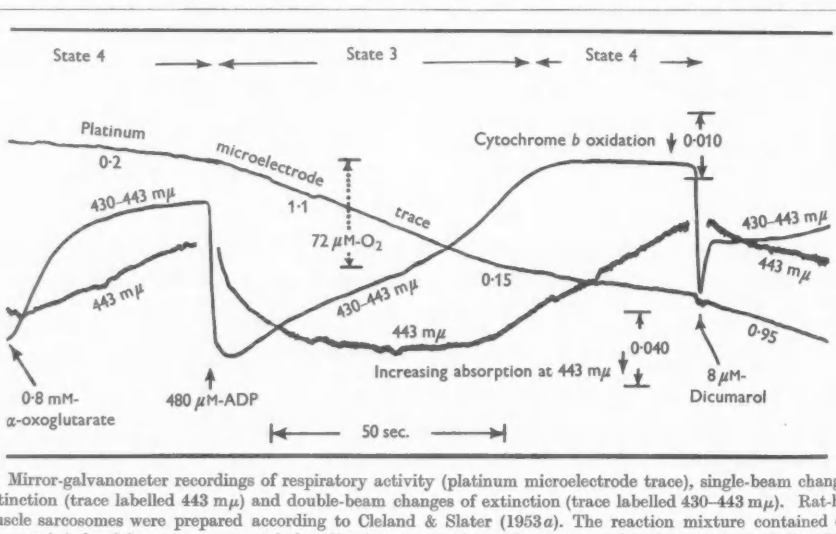

Fig. 1. Mirror-galvanometer recordings of respiratory activity (platinum microelectrode trace), single-beam changes of extinction (trace labelled 443 mμ) and double-beam changes of extinction (trace labelled 430–443 mμ). Rat-heart-muscle sarcosomes were prepared according to Cleland & Slater (1953*a*). The reaction mixture contained 4 mg. of protein/ml., 0·3 M-sucrose, mM-ethylenediaminetetra-acetic acid, 17·5 mM-phosphate, 8·8 mM-KCl, pH 7·5. Cuvette volume 2·5 ml. with a 1 cm. optical path. The molar concentrations of the reagents added are indicated on the figure and are converted into μmoles by dividing by 400. Additions: 1·2 μmoles of ADP (0·01 ml. of 0·12 M-ADP solution), 2 μmoles of α-oxoglutarate, 0·02 μmoles of Dicumarol. These additions cause negligible changes in tonicity. Respiratory rates (μM-O_2/sec.) and oxygen concentration changes during phosphorylation are indicated below the platinum microelectrode trace. The sensitivities of the recordings at 443 and at 430–443 mμ are indicated. An increase of absorption at 443 mμ is indicated by a downward deflexion of both traces. An upward deflexion of the 430–443 mμ trace indicates an increase of absorption at 430 mμ. Since the absorption band of cytochrome *b* disappears on oxidation, this is registered by a net downward deflexion of the 430–443 mμ trace. Temperature, 26°.

Non-specific light-absorption, measured at 443 mμ after the addition of α-oxoglutarate, shows an upward deflexion of the trace corresponding to a decrease of extinction. Such an extinction change is attributed to a swelling of the sarcosomes (Cleland & Slater, 1953*b*). Upon addition of ADP there is a large increase of extinction at 443 mμ indicative of a shrinking of the sarcosomes. However, this reaction is slow compared with the spectroscopic effect measured at 430 and 443 mμ. After ½ min. the extinction of the suspension becomes stable. When the added ADP is exhausted, as indicated by the slackening of respiration, the extinction at 443 mμ begins to decrease and continues to do so in the absence of ADP. Addition of Dicumarol stops this decrease and causes a slight increase of extinction.

The single- and double-beam recordings differ from each other in three ways. First, the single-beam recording has no critical wavelength dependency as shown by other experiments, whereas the double-beam recording shows a sharp wavelength dependency with a peak at 430 mμ [see Fig. 8 of Chance & Baltscheffsky (1958)]. Secondly, the kinetic effects differ: the double-beam recording shows a rapid response to the addition of ADP, whereas the single-beam recording responds slowly. (This is not an instrumental effect since both galvanometers have adequately rapid response to reproduce faithfully these reaction kinetics.) Conversely, the double-beam recording shows no change after the added ADP has been exhausted, and the single-beam recording shows a rapid decrease of extinction. Thirdly, the sign and magnitude of the effects are different. The addition of ADP causes a decrease of absorption at 430 mμ measured with respect to 443 mμ, whereas the single-beam record shows an increase of absorption at 443 mμ. Thirty seconds after the addition of ADP the latter record indicates an increase of extinction 3·7 times the decrease measured by the double-beam system. The major findings given here have been reproduced in a large number of experiments.

It is now possible to compare these results with those of Holton (1955), who has recorded the effect of ADP addition to heart-muscle sarcosomes with a single-beam technique at 433 mμ. From our data, about 75% of the spectroscopic change recorded with such a technique is a non-specific increase of absorption. Holton also finds an increase of absorption upon ADP addition, and at the end of about 2 min. he finds a slow decrease of absorption which corresponds closely to that shown in our Fig. 1, provided that we accept the very likely assumption that the 110 μM-ADP used in his experiment was expended at the end of 2 min. Thus the single-beam recordings of the effect of ADP obtained by Holton and by us are in good agreement. However, it is not justifiable to attribute the change of extinction caused by ADP addition to a specific spectroscopic effect. Thus his interpretation that cytochrome *b* is more reduced in the presence of ADP than in its absence under his experimental conditions appears to be incorrect.

These records permit us to correlate in a preliminary fashion the metabolic state of the sarcosomes and light-scattering changes. In the presence of α-oxoglutarate and in the absence of ADP (state 4), the sarcosomes appear to swell; in the presence of both α-oxoglutarate and ADP (state 3), the sarcosomes shrink rapidly at first and then become comparatively stable. It appears that the sarcosomes are less stable in quiescent state 4 than in the active state 3. Since adenosine triphosphate (ATP) is present in both states, it is suggested that 'high-energy' intermediates formed during oxidative phosphorylation are more useful in maintaining the sarcosomes in an unswollen state than is ATP itself.

SUMMARY

1. Simultaneous single-beam and double-beam recordings of extinction changes in a suspension of heart-muscle sarcosomes show that addition of adenosine diphosphate causes a non-specific increase of absorption (measured at 443 mμ) and a specific decrease of absorption at 430 mμ measured with respect to 443 mμ. The non-specific change is about four times as large as the specific one.

2. A single-beam spectrophotometric technique is not suitable for the measurement of spectroscopic effects in suspensions of sarcosomes and mitochondria that show simultaneous non-specific changes of light-absorption.

3. If it is assumed that the non-specific light-absorption properties of the sarcosomes are indicative of the ion-transport processes, these results suggest that intermediates formed during oxidative phosphorylation are more effective in preventing swelling of the sarcosomes than is adenosine triphosphate itself.

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The Chemistry of Connective Tissues

4. THE PRESENCE OF A NON-COLLAGENOUS PROTEIN IN CARTILAGE*

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Work carried out some years ago (Partridge, 1948*b*) showed that the intercellular matter of hyaline cartilage is composed in very large part of two main components: chondroitin sulphate and collagen. Recently, however, the presence of a non-collagenous protein in cartilage has been reported by Shatton & Schubert (1954), who showed that aqueous extraction of fresh bovine nasal cartilage at 1° released a quantity of material which behaved as a rather stable compound of the new protein and chondroitin sulphate and was described by the authors as a mucoprotein. Soon afterwards Muir (1956) observed a marked reduction in the viscosity of an undegraded preparation of chondroitin sulphate from laryngeal cartilage on treatment with papain and suggested that such preparations may consist of polysaccharide units cemented by protein.

The extraction of chondroitin sulphate from cartilage in an undegraded form is a problem which has attracted many workers. The experience in the past has been that in order to effect the release of polysaccharide in good yield it is necessary to employ rather vigorous reagents and these give rise to extensive degradation. Good yields of chondroitin sulphate of high chemical purity may be obtained by extraction with caustic alkali, but the molecular weight of such preparations is considerably lower than that of extracts prepared under milder conditions. If undegraded preparations are required it is necessary to employ neutral reagents such as calcium chloride (Blix & Snellman, 1945). However, most of the effective reagents of this type reduce the temperature of thermal shrinkage of collagen and bring a great deal of the protein into solution (Partridge, 1948*b*). The molecular weight of preparations of chondroitin sulphate, prepared by neutral extraction, has been given as 200 000–300 000 by Blix & Snellman (from viscosity measurements) and 150 000 by Matthews (1955, light-scattering), while preparations prepared by the use of caustic alkali have molecular weights estimated to be in the range 10 000–50 000 by Meyer & Odier (1946) and 18 000–48 000 by Matthews.

* Part 3: Partridge & Davis (1955).

A considerable advance in the technique of extraction was made when Einbinder & Schubert (1950) introduced a new reagent consisting of a 30% (w/v) aqueous solution of potassium chloride containing 1% of potassium carbonate. Use of this solution extracts a product of high viscosity in good yield, but yet prevents extensive solubilization of collagen. Preliminary experiments carried out in this Laboratory showed that extraction of finely ground cartilage powder with this mildly alkaline reagent was effective in separating the bulk of the polysaccharide-containing material from the residual collagenous structure without causing observable degradation, and it was thought that an extract so prepared would offer a suitable starting-point for a further study of the composition of the ground substance of this tissue.

EXPERIMENTAL

Preparation of the cartilage powder. Fresh nasal cartilage from a group of young cattle was freed from connective tissue and chopped finely with a scalpel. The pieces were rapidly washed in 1% NaCl solution, followed by water, and were then introduced at once into acetone. After standing in two or three changes of solvent the pieces were dried in air at 70° and then ground to a fine powder in a hammer mill.

Extraction with potassium chloride–potassium carbonate solution. The procedure followed generally the description given by Einbinder & Schubert (1950). For the first extract (extract *A*, Table 1) the cartilage powder (30 g.) was suspended in 500 ml. of a solution containing 300 g. of KCl and 10 g. of K_2CO_3/l. and shaken for 48 hr. at room temperature (18–20°). A second extract (*B*, Table 1) was obtained by shaking the residue in a further quantity (500 ml.) of the same solution for 96 hr. Finally two further extracts, designed to exhaust the cartilage, were prepared by extracting the residue for 48 hr. with 30% KCl containing 1% of KOH (extract *C*), followed by 48 hr. with 30% KCl containing 2% of KOH (extract *D*). Each of the extracts was dialysed against repeated changes of distilled water until free from chloride; after samples for determination of dry weight and total hexosamine (after hydrolysis) had been taken, the solutions were frozen at −10° for storage. Analysis of a typical series of extracts is given in Table 1.

Zone electrophoresis. The apparatus was based on the design of Haglund & Tiselius (1950). A vertical jacketed

column packed with graded potato starch according to the procedure of Carlson (1954) was arranged over an automatic fraction changer so that at the end of the experiment the separated zones could be forced off the column by the slow passage of buffer. Two columns of different size (30 cm. × 1·1 cm. and 30 cm. × 2·9 cm.) were in use in these experiments; both columns were provided with water jackets. Silver–silver chloride electrodes were employed similar to those used in the standard Tiselius (1937) moving-boundary apparatus. Details of the electrode vessels and connecting bridges will be given in a later publication.

To serve as a marker and as a control on the correct functioning of the column during elution, a small amount of bovine carbon monoxy haemoglobin was added to every sample; an experiment was accepted as satisfactory only if the red band due to carbon monoxy haemoglobin remained narrow and sharp after passage of the current, and if it were not distorted during the progress of elution. At pH 8·6 the migration of haemoglobin is very slow (about 0·05 cm./hr. at 8·5 v/cm.); it had the lowest mobility of any of the zones, and remained near the top of the column after passage of the current. During elution the arrival of the red band at the foot of the column was taken as an indication that the process was complete. For the separation of cartilage extracts the apparatus was used with water at room temperature, instead of the usual 2°, because the high viscosity of the extract at low temperature causes irregularity of electrophoretic migration and of elution.

A typical experiment with the 2·9 cm. diameter column was conducted as follows. A sample (2 ml.) of the freeze-dried extract (30 mg. in 1·5 ml. of buffer, to which was added 1·5 ml. of a 5% solution of carbon monoxy haemoglobin in the same buffer) was introduced on to the top of the column. The buffer used was sodium barbiturate (0·05 M) of pH 8·38 (glass electrode). The red haemoglobin band was first forced down the column to a position about 6 cm. from the top by application of buffer. The bridges connecting the electrode vessels were then put in place and the assembly filled with buffer. A potential of 400 v (12·4–12·8 mA) was then applied for 21 hr.; the bath temperature rose slowly from 21·8° to 23·8° during the course of the run. Through the combined effects of electrical transport and endosmosis the haemoglobin band migrated slowly towards the negative pole, finishing just below the top surface of the column at the end of the run. The bridge at the bottom of the column was then disconnected and buffer was applied from a reservoir arranged at a height sufficient to give a flow through the column of 5–10 ml./hr. Some forty fractions were taken at 10 min. intervals, up to the time of breakthrough of the red haemoglobin solution.

Estimation of hexosamine in the fractions. Since the analysis of some forty fractions was required for each experiment, it was necessary to adopt a simple and rapid procedure for hydrolysis. Complete hydrolysis or optimum colour yields was not required, the main object of the determination being to establish the position and shape of any zones due to hexosamine-containing polysaccharides. After preliminary trials the following procedure was adopted. Samples of solution (0·2 ml.) were taken from each fraction and introduced into a set of soda-glass test tubes, matched for photometric readings. 5 N-Hydrochloric acid (0·2 ml.) was then added to each. The tubes, supported in a rack and closed by means of glass bulbs, were then immersed in a boiling-water bath for 4 hr., at least two-thirds of each tube projecting from the water to provide condenser action. When it was cool, the solution was brought to near neutrality by the addition of 0·2 ml. of 4·8 N-NaOH, care being taken not to render the solution alkaline. Estimation of hexosamine was then continued with the same tubes by following the method of Elson & Morgan (1933) as modified by Blix (1948).

Estimation of protein in the fractions. Protein was estimated colorimetrically by the modified biuret method of Lowry, Rosebrough, Farr & Randall (1951), a commercial gelatin being used as the standard.

Other analyses carried out on the fractions. For the purpose of following the separation of the protein and polysaccharide components the estimation of hexosamine and 'total protein as gelatin' by the methods given above gave enough information. However, in a few of the experiments neutral polysaccharides were looked for by examining each fraction for 'sugars other than hexosamine' by the anthrone method of Dreywood (1946) as modified by Yemm & Willis (1954). In some experiments the fractions were also examined for uronic acid by the method of Anderson & Maclagan (1955), with galacturonic acid as a standard. The results obtained agreed with the view that the only major components of the system were proteins of collagenous and non-collagenous origin, together with chondroitin sulphate.

Removal of soluble collagen by treatment with an ion-exchange resin. The resin used was the fine-grade form (XE-64) of the carboxylic acid cation exchanger, Amberlite IRC-50 (obtainable from Charles Lenning and Co., London). Particles in the 150–200 mesh range were obtained by removing the fines. This was done by suspending the resin in water, allowing it to settle for 30 min. and removing the supernatant solution. The resin was passed twice through the sodium form by alternate treatments with 2 N-NaOH and 2 N-HCl, the resin being washed with distilled water between each treatment. The resin was finally air-dried in the H^+ ion form for storage.

The purified resin (1·2 g.) was suspended in 0·2 M-potassium acetate buffer of pH 5·0, allowed to settle and the supernatant removed. The process was then repeated until the pH of the supernatant was the same as that of the buffer. The dialysed cartilage extract (40 ml., containing about 1% of total solids) was mixed with 10 ml. of M-acetate buffer of pH 5. The equilibrated resin was then added and the mixture shaken gently for 30 min., after which the resin was collected in the centrifuge and washed with diluted buffer and the washings were added to the supernatant.

The resin was extracted three times with 1% (w/v) K_2CO_3 by stirring and centrifuging. The first extract required the addition of a few drops of 2 N-KOH to bring the mixture to about pH 10 before centrifuging. The alkaline extract from the resin contained protein, which from its hydroxyproline content was of collagenous origin. The protein was recovered by neutralizing the solution, and evaporating to small bulk after thorough dialyses against distilled water. The protein was then freeze-dried.

After extraction of the protein the resin was brought to the H^+ ion form and again equilibrated with buffer of pH 5. The cartilage extract was then treated again with the same batch of resin. The first treatment with resin removed 90–95% of the hydroxyproline-containing protein from the cartilage extract and the second treatment removed the remainder. Since it was found that components in the

cartilage extract other than hydroxyproline gave rise to a colour with the dimethylaminobenzaldehyde reagent of Newman & Logan (1950) this method was unsuitable for the estimation of the small amounts of collagenous protein which remain after resin treatment (cf. Miyada & Tappel, 1956). Removal of collagen after the treatments with the resin was therefore finally checked by showing the absence of a peak due to hydroxyproline in an elution chromatogram of the hydrolyses products carried out by the method of Moore & Stein (1951). As recommended by these authors, the pH of the sodium citrate buffer used for elution was lowered slightly to pH 3·04 in order to secure sharp separation of hydroxyproline from aspartic acid.

Isolation of the non-collagenous protein prepared by acid hydrolysis. A cartilage extract that had been treated with resin to remove collagenous protein as above was dialysed against several changes of distilled water to remove buffer salts. The solution was then concentrated to approximately 2% of solids by use of rotary evaporator (Partridge, 1951). To the concentrated solution (273 ml. containing 5 g. of solids) 4 ml. of acetic acid and 40 ml. of 1% (w/v) picric acid solution were added. The solution was then heated on a water bath, with an air condenser, for 6 hr. On cooling, the yellow flocculent precipitate of protein was separated by centrifuging, leaving a clear yellow supernatant. The supernatant solution was again heated for 3 hr. at 90°, when it developed a further light precipitate. This was removed by filtration and discarded. The precipitated protein was washed with dilute acetic acid–picric acid mixture, and dissolved in water with the addition of a few drops of NaOH solution to pH 9. It was then precipitated by adding 5 vol. of ethanol and adjusting the pH to 5 by the addition of acetic acid. This operation was repeated 2–3 times until the precipitate was colourless. In this condition the protein could be recovered quantitatively from alkaline solution in water by the addition of acetic acid to pH 4. The protein was finally washed with ethanol and 50% (w/v) ethanol–ether mixture and dried to a fine white powder (0·27 g.) under vacuum at 40°. Analyses carried out with this preparation are recorded in Tables 2 and 3.

Isolation of chondroitin sulphate prepared by acid hydrolysis. The supernatant from picric acid treatment was brought to pH 5 and 6 vol. of ethanol added. After standing overnight the precipitate was collected by centrifuging. The precipitate was then redissolved in 100 ml. of water and again precipitated with ethanol; a few crystals of potassium acetate were added to cause the precipitate to coagulate. The process was repeated twice more until the precipitate was colourless. The yield of crude polysaccharide was 3·4 g. Hydrolysis and two-dimensional paper chromatography [solvents: phenol–aq. NH_3 and butanol–acetic acid (Partridge, 1948*a*)] of a sample of the crude polysaccharide showed that amino acids were still present. Since this may have been due to uncombined protein or peptides which had escaped precipitation with picric acid, the solution was treated with the carboxylic acid resin at pH 5 according to the procedure described above. The supernatant solution was dialysed against four to five changes of distilled water and the polysaccharide recovered by ethanol precipitation with the addition of a little potassium acetate. The precipitate, which was then free from amino acids after hydrolysis, was redissolved in about 50 ml. of water, dialysed against distilled water to remove potassium acetate and freeze-dried.

Isolation of the non-collagenous protein prepared by alkaline hydrolysis. A preparation (4·5 g.) of the collagen-free protein–polysaccharide complex (preparation C, Table 2) which had been freeze-dried was dissolved in 0·5N-KOH (200 ml.). The solution was allowed to stand in a constant-temperature room at 25° with occasional gentle shaking, and after 20 hr. was neutralized to pH 5·0 by the addition of acetic acid. The solution was then diluted to 1 l. and shaken for 2 hr. with 10 g. of IRC-50 resin, purified and equilibrated with buffer of pH 5 as described above for the removal of collagen. The resin was separated by centrifuging and the solution again shaken with a second 10 g. quantity of resin.

The two batches of resin were mixed, and washed on the centrifuge four times with 0·1 M-potassium acetate buffer of pH 5·0 (300 ml.), the washings being discarded. The resin was then suspended in water, and dilute KOH added dropwise to the stirred slurry to bring it to pH 8–9. A few millilitres of 1% (w/v) K_2CO_3 solution were then added and the mixture was centrifuged. The resin was extracted twice more with 1% (w/v) K_2CO_3 solution and the mixed supernatant collected for recovery of the protein. The supernatant solution was neutralized to pH 5–6 with acetic acid and dialysed against distilled water until the diffusate was free from potassium. The solution was then concentrated to small bulk at 40° and freeze-dried. The yield was 0·12 g.

Isolation of chondroitin sulphate prepared by alkaline hydrolysis. The hydrolysis product, after treatment with resin, contained chondroitin sulphate substantially free from protein. A further treatment with resin was given to ensure complete removal of protein, and the supernatant, which was a pale-amber colour, was concentrated to 500 ml. and dialysed against distilled water. It was then further concentrated to 125 ml. and adjusted to pH 7 with a few drops of KOH solution. The solution was again dialysed against several changes of distilled water until the diffusate contained less than 1·5 p.p.m. of K^+ ions. The product (3·2 g.) was freeze-dried.

Determination of hexosamine in purified preparations of chondroitin sulphate. Optimum conditions for the hydrolysis of chondroitin sulphate were investigated by hydrolysing samples in HCl of various concentrations and for varying lengths of time. The polysaccharide proved to be more resistant to hydrolysis than many mucoids, and in order to complete the reaction in a reasonable time it was necessary to use the acid at a rather high concentration. Employing 4N-HCl at 100°, maximum colour yield with the Elson & Morgan (1933) reagents was obtained after 16 hr. hydrolysis, and thereafter the colour yield fell slowly (cf. Smith & Zwartouw, 1956). Both glucosamine and galactosamine are slowly destroyed when they are maintained at 100° in the presence of 4N-HCl, but whereas with glucosamine only about 5% was lost after 16 hr. treatment (cf. Boas, 1953) the loss with a pure sample of galactosamine proved to be 10–14% in different experiments. When equimolecular solutions of galactosamine and galacturonic acid were heated with HCl under the same conditions the loss of galactosamine was about the same.

RESULTS

Extraction of the cartilage powder. A series of preliminary experiments were carried out on the course of extraction of material containing hexos-

amine by 30% (w/v) potassium chloride solution containing alkali. The results of extraction at 18–20° are summarized in Table 1. Of the hexosamine available in the cartilage 56% was extracted into KCl–K_2CO_3 solution over a period of 6 days. Towards the end of this period extraction became very slow, and more alkaline solutions were needed to release the remainder of the hexosamine-containing material. Final exhaustion of the cartilage powder was obtained by employing 30% potassium chloride solution containing 2% (w/v) of potassium hydroxide; but the last extract contained only 1·9 g. of hexosamine/100 g. of dry solids, showing that these severely alkaline conditions resulted in extensive dissolution of collagen.

Column 7 of Table 1 shows that the successive extracts gave material which was progressively poorer in hexosamine, and since the aim was to isolate the components of the tissue which contain a high proportion of polysaccharide a single 48 hr. extract in potassium chloride–potassium carbonate solution was employed for further study. Table 2 gives analyses of material extracted into potassium chloride–potassium carbonate solution at room temperature (extract A) and at 0° (extract B). Judged by the hydroxyproline contents, extract A contained about 39% of protein derived from collagen, whereas B contained only 10%. All subsequent experiments were therefore carried out with material extracted at 0°.

Zone electrophoresis. Fig. 1 (*a*) shows the zone-electrophoresis pattern given by extract B (Table 2). The points representing hexosamine content (solid circles) were determined after partial hydrolysis (see experimental section) and the values, while showing the position of the zones due to chondroitin substrate, do not have quantitative significance. It will be seen that while part of the protein (peak *Z*) migrated only slowly in the electric field fractions 1–15 (peaks *X* and *Y*), which contained hexosamine, also contained protein. The pooled fractions containing hexosamine were found to be free from hydroxyproline after hydrolysis, showing that the protein which migrated with chondroitin sulphate was not of collagenous origin. Fig. 1 (*b*) shows the zone-electrophoresis pattern obtained under the same experimental conditions with the same batch of extract after treatment with 0·5N-sodium hydroxide for 4 hr. at 25°. The alkali-degraded material separated sharply into two zones, one of which (peak *X*) contained polysaccharide uncontaminated with

Table 1. *Yields and analytical data of products from successive extracts of* 30 *g. of dry cartilage powder*

Method of extraction	Extract	Vol. of extract (ml.)	Total solid (by dry wt., g.)	Hexosamine (mg./ml. of extract)	Total hexosamine in extract (g.)	Hexosamine in extracted solid (%)	Proportion of original hexosamine extracted (%)
48 hr. 30% KCl–1% K_2CO_3	*A*	500	3·45	0·875	0·428	12·7	37·7
96 hr. 30% KCl–1% K_2CO_3	*B*	550	1·97	0·387	0·212	10·8	18·3
48 hr. 30% KCl–1% KOH	*C*	490	3·76	0·66	0·324	8·6	28·0
48 hr. 30% KCl–2% KOH	*D*	610	4·78	0·149	0·091	1·9	7·8
Total	—	—	13·96	—	1·055	—	91·8
Residue	—	—	—	—	0·051	—	4·4

Table 2. *Composition of preparations from ox cartilage* (septum nasi)

	Nitrogen (%)	Hexosamine (%)	Ash as K_2SO_4 (%)	Hydroxyproline (%)
A. Material extracted into KCl–K_2CO_3 solution 48 hr. at 18–20°	10·04	11·6	13·1	5·5
B. Material extracted into KCl–K_2CO_3 solution 48 hr. at 0°	6·17	18·3	22·5	1·44
C. Extract B after treating with resin to remove collagen	4·13	24·0	18·6	Nil
D. Non-collagenous protein prepared by acid hydrolysis of C	13·7	6·9	5·5	Nil
E. Non-collagenous protein prepared by alkaline hydrolysis of C	13·3	2·03	0·5	Nil
F. K salt of chondroitin sulphate prepared by acid hydrolysis of C	2·8	27·4	29·9	—
G. K salt of chondroitin sulphate prepared by alkaline hydrolysis of C	2·8	27·2	30·2	—
H. Calc. for chondroitin sulphate, $C_{14}H_{19}O_{14}NSK_2$	2·62	33·5	32·5	—

protein. This is in accord with the usual experience that alkali-treated or alkali-extracted chondroitin sulphate can readily be separated from protein by purely physical methods.

It will be observed that in Fig. 1 (*a*) the composite peak $X+Y$ is not symmetrical and is not uniform in composition. The shape of the composite peak suggests that it has resulted from the summation of two components of high mobility, the slower-moving of the two migrating as a rather broad zone and containing most or all of the protein. The faster-moving component may be identified, as regards position, with peak X in Fig. 1 (*b*) and may be due to free chondroitin sulphate. The slower-moving component (Y) carries the non-collagenous protein and disappears after hydrolytic treatment with sodium hydroxide: it is therefore considered to be due to a protein–polysaccharide complex or mucoprotein. The small protein peak in Fig. 1 (*b*) covering fractions 10–15 may be a residue of the mucoprotein which has escaped hydrolysis. Peak Z in Fig. 1 (*a*) contained hydroxyproline in the proportion which would be expected if it were derived from collagen and the peak was therefore identified with this protein. Peak Z in Fig. 1 (*b*) was considerably increased in amount after the hydrolytic treatment and is regarded as consisting of a mixture of collagenous and non-collagenous protein.

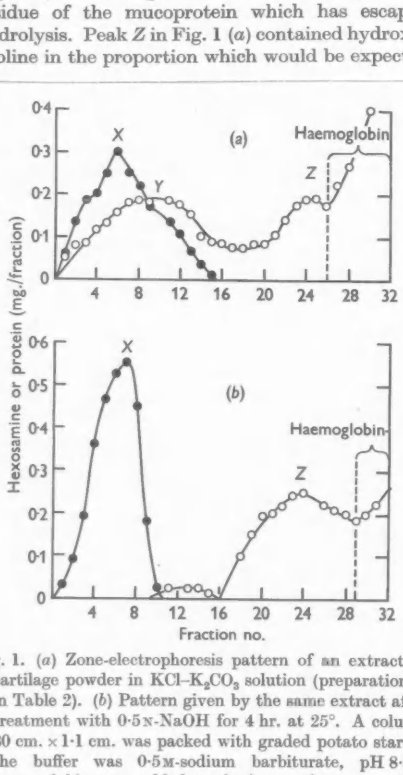

Fig. 1. (*a*) Zone-electrophoresis pattern of an extract of cartilage powder in KCl–K_2CO_3 solution (preparation B in Table 2). (*b*) Pattern given by the same extract after treatment with 0·5 N-NaOH for 4 hr. at 25°. A column 30 cm. × 1·1 cm. was packed with graded potato starch; the buffer was 0·5 M-sodium barbiturate, pH 8·54; haemoglobin was added to both samples as marker. ○, Protein estimated against a gelatin standard (Lowry *et al.* 1951); ●, hexosamine estimated after partial hydrolysis (Elson & Morgan, 1933).

Isolation of the mucoprotein. The interpretation of the zone-electrophoresis experiments given above was confirmed by the isolation of the polysaccharide–protein complex assumed to be in admixture or loosely bound with soluble collagen. Boardman & Partridge (1955) showed that below pH 6·5 many proteins were very strongly adsorbed on the fine-mesh form of the carboxylic acid resin IRC-50, and most could readily be desorbed again by treatment with alkaline buffers. It was found that a single treatment of the cartilage extract (B in Table 2) with the resin removed 90–95 % of the hydroxyproline-containing protein and that two treatments completed the removal. The mucoprotein complex which remained contained N, 4·13; hexosamine, 24·0 %; and its protein content was thus about 10 %. Since 3–4 further treatments with ion-exchange resin failed to reduce the nitrogen content of the complex it seemed clear that the non-collagenous protein was firmly bound and possibly in chemical combination with the polysaccharide. An attempt was made to dissociate the mucoprotein by mild hydrolysis, and a series of trials showed that the complex readily yielded to degradation with dilute organic acids at 95° or dilute aqueous sodium hydroxide at 25°.

Hydrolysis with acid. On heating solutions of the mucoprotein in 1 % acetic acid solution, the slightly opalescent solution became increasingly opaque and addition of picric acid resulted in the immediate separation of a flocculent yellow precipitate. It proved convenient to carry out the hydrolysis in a mixture of picric acid and acetic acid, when the bulk of the liberated protein could be removed as insoluble picrate after about 6 hr. at 95°, when 80–90 % of the reaction was complete. Longer periods of heating resulted in the gradual destruction and resolution of the protein picrate.

The crude protein, after removal of picric acid, had N, 13·7; ash, 5·5 %; P, absent. It dissolved to form a clear colourless solution in aqueous sodium hydroxide and was precipitated quantitatively by adjustment of the pH to 4·5. Determination of the hexosamine content (colorimetric) gave 6·9 % of hexosamine, showing that the liberation of the polysaccharide moiety was not complete. No conditions for the acid hydrolysis of the mucoprotein could be found which would result in the clear-cut separation of the protein in high yield, and all preparations contained some polysaccharide. In Table 3 a partial amino acid analysis of a preparation of the crude protein is given (method of Moore & Stein, 1951).

Table 3. *Amino acid composition of the protein moiety of the mucoprotein compared with ox-bone collagen*

	Prepared by acid hydrolysis of the mucoprotein		Prepared by alkaline hydrolysis of the mucoprotein		Ox-bone collagen analysis by Eastoe (1955)
	g. of N/100 g. of protein N	g. of amino acid/100 g. dry wt.	g. of N/100 g. of protein N	g. of amino acid/100 g. dry wt.	g. of amino acid/100 g. dry wt.
Hydroxyproline	0·03	0·04	Nil	Nil	14·1
Aspartic acid	6·12	7·96	5·71	7·3	7·1
Threonine	2·74	3·19	2·86	3·27	2·52
Serine	3·16	3·25	2·64	2·65	4·24
Glutamic acid	7·0	10·0	8·80	12·4	11·9
Proline	5·37	6·05	6·94	7·86	14·7
Glycine	7·55	5·54	4·79	3·46	25·3
Alanine	4·88	4·24	4·79	4·11	10·5
Valine	3·92	4·49	4·05	4·54	2·65
Methionine	0·84	1·23	0·87	1·22	0·80
Isoleucine	3·05	3·91	2·77	3·50	1·73
Leucine	6·60	7·69	6·15	7·73	3·93
Tyrosine	2·47	4·38	2·60	4·54	0·56
Phenylalanine	3·17	5·12	4·67	7·42	2·88
Histidine	—	—	3·67	1·83	0·96
Lysine	—	—	5·04	3·50	4·11
Arginine	—	—	5·57	2·3	9·2
Glucosamine	—	—	0·35	0·61	—
Galactosamine	—	—	0·33	0·56	—
Unknown amino sugar	—	—	—	0·33	—

Crude chondroitin sulphate was recovered from the supernatant solution by precipitation with ethanol and the product purified by further reprecipitation. The analysis of a typical sample is given in Table 2. The value for nitrogen (2·84%) indicated that the protein content of the material was small and this was confirmed by paper chromatograms carried out on hydrolysed samples. These showed only traces of two or three amino acids.

Hydrolysis with alkali. The polysaccharide prepared by treatment for 4 hr. under the conditions given had N, 3%; since paper chromatograms carried out after hydrolysis with 5·5N-hydrochloric acid showed the presence of spots due to seventeen amino acids, it was concluded that the preparation still contained mucoprotein and that alkaline hydrolysis was incomplete. Analysis of the 20 hr. and 40 hr. preparations showed them to be almost identical in composition, both having N, 2·8; hexosamine, 26·3%. Samples of both preparations were hydrolysed and tested for the presence of amino acids by paper chromatography. Apart from the very large spots due to chondrosamine, only one or two traces of ninhydrin-reactive substances were observed, and the preparations were regarded as substantially free from protein.

Further purification of the polysaccharide from a 40 hr. alkaline hydrolysis was attempted by employing a solvent-fractionation procedure. A centre fraction from ethanol precipitation was refractionated by step-wise addition of 90% (w/v) aqueous phenol. The analysis of the centre fraction from phenol precipitation is given in Table 2 (preparation G) and shows little change in composition from the crude material.

Purified samples of chondroitin sulphate prepared by acid and alkaline degradation had almost identical composition, but the analytical results show that the integral ratios (g.atom or g.mol./g.mol. of $C_{14}H_{19}O_{14}NSK_2$) for nitrogen, hexosamine and potassium sulphate deviated from unity; these ratios were 1·08, 0·82 and 0·92 respectively. The high value for nitrogen is understandable and may be due to contamination with traces of peptides, but the low hexosamine figure is outside the range of expectable error. Less than unit integral ratios for hexosamine have been reported by other workers. Thus Einbinder & Schubert (1950) gave hexosamine contents averaging at 23·6%, whereas Blix & Snellman (1945) reported 24–25% for highly purified preparations; on the other hand, Meyer & Smyth (1937), analysing the acid calcium salt, gave higher figures (28–30%). All the galactosamine determinations reported here have been carried out with material hydrolysed for 16 hr. with 4N-hydrochloric acid at 100° and, as indicated in the Experimental section, the results may be assumed to be about 12% low owing to loss during hydrolysis. Correction for this loss brings the integral ratio for galactosamine to 0·93, a figure which agrees well with the corresponding ratio (0·92) for potassium sulphate derived from estimation of the ash. Chromatographic analysis of the hydrolysis products of both preparations of chondroitin sulphate (F and G in Table 2) by the procedure of Gardell (1953) showed the absence of amino sugars

other than galactosamine, and the quantitative recoveries were identical with those obtained by direct colorimetric estimation. The analytical results confirm the accepted formulation for chondroitin sulphate and suggest that, apart from a small contamination with material of peptide character (and possibly with bound water), the preparations were substantially pure.

The protein which was released during alkaline hydrolysis was recovered from the ion-exchange resin by elution with alkaline buffer after exhaustive washing. The analysis of a preparation from a 40 hr. hydrolysate is given in Table 2 (preparation E). The protein contained 2·03% of hexosamine (colorimetric) and 0·5% ash, showing that even after prolonged hydrolytic treatment some mucoprotein was still present. A complete amino acid analysis (obtained by the method of Moore & Stein, 1951) is given in Table 3 (main column 2). Hydroxyproline is absent and the proportions of the other amino acids are very different from those found in collagen (Table 3, main column 3). The high content of tyrosine (4·54%) in the new protein is of particular interest. Ion-exchange chromatography of the protein hydrolysate carried out according to the method of Gardell (1953) showed three peaks due to material reacting with the Elson & Morgan (1933) reagent. The first peak (fractions 74–78) was due to an unknown substance which, estimated as a hexosamine against a glucosamine standard, represented 0·33% of the protein dry weight. The second peak (fractions 81–86) was glucosamine (0·61%) and the third (fractions 98–102) was galactosamine (0·56%). Since pure chondroitin sulphate contains no glucosamine it is clear that a large part of the polysaccharide content of the protein must have another origin. Glucosamine was found in all preparations of the protein, whether from acid or from alkaline degradation or the original mucoprotein, but its origin remains unknown. The nitrogen content of the protein is very low (about 13·5%) and only 68% of the nitrogen was recovered as amino acids in the Moore & Stein chromatograms. This indicates that only about two-thirds of the substance is of polypeptide character and that the remainder of the material must contain nitrogenous and non-nitrogenous components other than amino acids. The amount of material obtainable thus far has been insufficient for further analysis, but work on the problem is continuing.

DISCUSSION

The discovery by Shatton & Schubert (1954) of a hitherto unrecognized protein component in combination with chondroitin sulphate in cartilage is clearly of importance in any attempt to understand the structure of the tissue, and the object of the work now described was to confirm and extend this observation by the isolation of the complex and the examination of its degradation products. From the outset it appeared unlikely that all the polysaccharide could exist in chemical combination with protein, since many workers have isolated chondroitin sulphate of quite high purity from cartilage by partial extraction under conditions which would not be expected to degrade a stable compound (see particularly Blix & Snellman, 1945). However, in these experiments the yields of pure polysaccharide have always been low. Much higher yields of pure material are obtainable by extraction with sodium hydroxide (cf. Meyer & Odier, 1946) but here the conditions employed are sufficiently hydrolytic to account for the dissociation of any pre-existing mucoprotein. High yields of free chondroitin sulphate may also be obtained if the fresh tissue is allowed to autolyse for some time before extraction (Einbinder & Schubert, 1950).

The evidence that part at least of the chondroitin sulphate in cartilage is in firm combination with a non-collagenous protein is derived from two independent types of experiment. These were the isolation of the complex by use of an ion-exchange resin and zone-electrophoresis experiments carried out on the degraded and undegraded extracts. When the initial extract was treated with an ion-exchange resin under conditions which were known to result in the adsorption of free protein, collagen was removed quantitatively but the remaining material still contained a protein which was free from hydroxyproline. Treatment of this residue with sodium hydroxide solution at room temperature effected the release of the non-collagenous protein, and when the solution was re-treated with the same resin the liberated protein was adsorbed, leaving chondroitin sulphate in a substantially pure form. The non-collagenous protein was then recovered by eluting it from the resin with alkaline buffer, and amino acid analysis confirmed that its composition was quite different from that of collagen. The zone-electrophoresis experiments carried out on the initial extract showed that the non-collagenous protein migrated with chondroitin sulphate as a zone of high mobility which was well separated from the zone due to collagen. A quite different pattern was obtained when extracts which had been treated with dilute aqueous sodium hydroxide were submitted to electrophoresis under the same experimental conditions. The chondroitin sulphate was then found to migrate as a sharp zone free from protein and the slower-moving material consisted of a mixture of collagen with the non-collagenous protein.

The results of electrophoresis in free solution (Partridge, 1948*b*) had previously indicated that in

undegraded extracts soluble collagen exists as an equilibrium mixture with chondroitin sulphate and a loose complex formed between the two. It was not then observed that chondroitin sulphate was itself combined with a second protein. Since it has been shown that the high viscosity associated with 'undegraded chondroitin sulphate' is rapidly reduced by the action of commercial trypsin (Shatton & Schubert, 1954) or papain (Muir, 1956), it appears that the protein content of these preparations is concerned in the formation of molecular aggregates. Preparations of chondroitin sulphate lose their capacity to combine reversibly with collagen when they are degraded by heating or by the action of dilute alkali (Partridge, 1948*b*). It therefore seems probable that the species responsible for the linkages with collagen that determine the integrity of the native tissue is the protein–polysaccharide macromolecule and not free chondroitin sulphate.

The possibility that the mucoprotein arises as an artifact during the process of extraction must be considered. Although cartilage from nasal septum contains only a small number of cells, these could themselves be a source of non-collagenous protein, and it is possible that such a protein could become associated with chondroitin sulphate as a result of the disorganization arising from extraction. However, this seems to be unlikely in view of the indications that the protein is chemically combined. Intervention of plasma proteins also seems to be unlikely in view of the small size of the free pore spaces in the cartilage gel. These have been given by Paulson, Sylvén, Hirsch & Snellman (1951) as of the order of 10–15 Å in bovine nucleus pulposus, suggesting that this system is permeable to simple sugars and amino acids but is impermeable to large molecules.

SUMMARY

1. The product of extracting cartilage powder prepared from bovine *septum nasi* with 30% potassium chloride–1% potassium carbonate solution has been examined.

2. Zone electrophoresis showed the extract to contain soluble collagen, a mucoprotein complex consisting of chondroitin sulphate combined with a non-collagenous protein and probably some free chondroitin sulphate.

3. The extract was freed from collagen by treatment with a carboxylic acid ion-exchange resin at pH 5.

4. Mild hydrolysis of the collagen-free material with dilute aqueous potassium hydroxide at 20° or acetic acid–picric acid mixture at 95° dissociated the complex and liberated the non-collagenous protein.

5. After release by alkaline hydrolysis, the new protein became available for adsorption on the same resin as was used for removal of collagen, and could be recovered from the resin by elution at pH 9.

6. The amino acid composition of the non-collagenous protein is given.

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The Determination of Uric Acid in Biological Materials

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Uric acid is commonly determined in biological fluids by measuring the blue colour produced by its reaction with reagents containing phosphotungstic or arsenotungstic acids under conditions which minimize interference by other reducing substances (Folin & Denis, 1912; Folin, 1933, 1934; Benedict, 1912, 1922; Brown, 1945; Bidmead, 1951). Recovery of uric acid added to blood has never been complete and values as low as 50 % have been recorded. Substances other than uric acid which react with the reagents occur in blood, notably ergothioneine (Hunter & Eagles, 1925) which occurs mainly in the erythrocytes. Benedict & Behre (1931) found that ergothioneine is removed when silver chloride is precipitated in the solution. Treatment with the enzyme uricase has been used to distinguish uric acid from other reacting substances (Blauch & Koch, 1939; Bulger & Johns, 1941; Block & Geib, 1947; Johnstone, 1952). The present paper describes experiments which have led to a satisfactory method for the assay of uric acid with a phosphotungstic acid reagent.

MATERIALS AND METHODS

AnalaR reagents were used where available. Sodium tungstate was tested by the xanthate reaction for molybdate and purified where necessary. The phosphotungstic acid reagent was prepared by the method of Folin (1934). Batches vary slightly in chromogenic activity and so one batch was used for each set of comparative experiments. The reagent yielded reproducible results over several months. The sodium cyanide–urea reagent was prepared as one solution from assayed sodium cyanide and was stored at 2°. Standard uric acid solution (1 mg./ml.) was prepared by the method of Folin (1930) and diluted as required. The method of Folin (1933) was used as the basic procedure, with 1 ml. of uric acid solution, 0·5 ml. of phosphotungstic acid reagent and 2 ml. of 5 % (w/v) sodium cyanide containing 15 % (w/v) of urea. The reaction time was 20 min. at 20°. The optical density of the blue reaction mixture was measured at 690 mμ with a Unicam spectrophotometer and 1 cm. light path.

RESULTS

Concentration of reactants. Experiments were carried out to determine the optimum proportions of reactants and the time and temperature for development of the colour. Greater colour production was achieved when the concentration of phosphotungstate reagent was increased, and standard curves were linear up to 25 μg. of uric acid in the reaction mixture with final concentrations of the reagent of 14 and 19 % (v/v). At 9 % concentration the curve was linear up to 10 μg. of uric acid. Standard curves were linear for final concentrations of sodium cyanide between 1·1 and 3·4 % (w/v). Maximum colour is produced with a final concentration of sodium cyanide between 2·3 and 3·1 % (w/v); a further increase yields less colour (cf. Fig. 3, broken line). Beyond 4·5 % (w/v) of sodium cyanide cloudy reaction mixtures are obtained. In all the experiments recorded 15 % (w/v) urea was incorporated in the sodium cyanide solution, but the amount of urea does influence the colour. A steady increase of 11·7 % in colour production was observed as the concentration of urea was increased from 7·5 to 22·5 %.

Time and temperature. Fig. 1 indicates that at elevated temperatures colour production is more rapid and some colour destruction takes place. The time required to reach maximum optical density depends on the temperature and is more prolonged at lower temperatures. Blank values rise rapidly

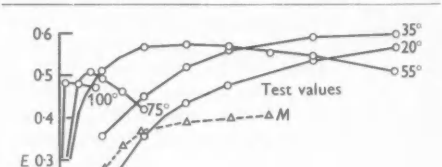

Fig. 1. Optical density produced from 1 ml. of uric acid solution (5 μg./ml.) when treated with 2 ml. of 5 % (w/v) sodium cyanide containing 15 % (w/v) of urea and 0·5 ml. of phosphotungstate reagent at given temperatures as a function of time. Blank values were obtained by substituting water for the uric acid solution. Curve *M* is given by the modified method described in the text.

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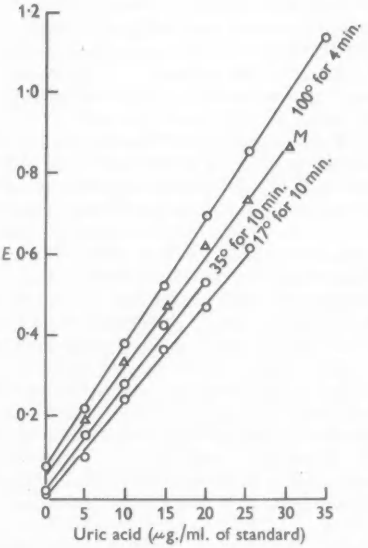

Fig. 2. Optical density produced by treatment of a series of uric acid standards (1 ml.) with 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea and 0·5 ml. of phosphotungstate reagent at different time and temperature combinations. Curve *M* is given by the modified method described in the text.

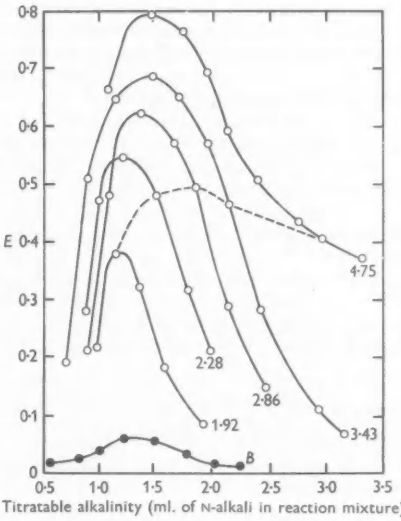

Fig. 3. Effect of modifying the alkalinity of the sodium cyanide–urea solution on the optical density of the final reaction mixture. Volumes of 5N-NaOH or 5N-H_2SO_4 were diluted to 0·5 ml. with water, mixed with 0·5 ml. of a uric acid standard containing 5 μg. and treated with 2 ml. of sodium cyanide solution containing 15% (w/v) of urea and 0·5 ml. of phosphotungstate reagent at 35° for 30 min. The final concentration of sodium cyanide (w/v) in the reaction mixture is indicated on the curves. The broken line joins points where neither acid nor alkali was added. Curve *B* gives blank values obtained by substituting water for uric acid and with a final concentration of sodium cyanide of 3·43% (w/v).

with increase in temperature. To test if the rate of colour production was proportional to the concentration of uric acid throughout the reaction period standards were treated at the times and temperatures shown in Fig. 2. Curves similar to those of Fig. 1 were obtained with the methods of Brown (1945) and Bidmead (1951). Maximum optical densities were less than with the reagent of Folin (1934) for the same amount of uric acid, colour destruction was more rapid and standard curves were not linear above 10 μg. of uric acid/ml. of test solution.

Sodium cyanide–urea reagent. When 2 ml. of a 5% solution of sodium cyanide is diluted to 10 ml. with water and titrated with N-H_2SO_4 the best grades show a titratable alkalinity of 1·85 ± 0·02 ml. Modification of the titratable alkalinity influences the colour yield with uric acid and the phosphotungstate reagent considerably, as illustrated in Fig. 3. Different batches of sodium cyanide gave widely different colour yields when tested with uric acid and the phosphotungstate reagent under identical conditions, as shown in Table 1, which also gives values for titratable alkalinity and assay of cyanide.

Table 1. *Optical density produced with different samples of sodium cyanide*

The basic procedure was used, with 5 μg. of uric acid.

	Sodium cyanide		
Description	NaCN content (%)	Titratable alkalinity (ml. of N-acid/0·1 g.)	$E_{690\,m\mu}$
Laboratory reagent			
1	78·1	1·61	0·492
2	93·3	1·85	0·540
3	92·4	1·85	0·335
4	93·1	1·86	0·321
Technical grade	61·8	1·33	0·083

Recovery of uric acid. Tungstic acid filtrates of serum prepared by the methods of Folin & Wu (1919) and of Folin (1930) with both slow and rapid addition of acid yielded identical uric acid assays. When uric acid was added to serum or to filtrates from the same serum recoveries were of the same

order. These findings indicate that loss of uric acid by co-precipitation with proteins is an improbable source of error in assays of uric acid in serum. The recovery of uric acid added to normal sera by three different techniques is given in Table 2.

Interfering substances. Investigation into the effects of interference in the colour reaction by a wide range of organic and inorganic compounds showed that the amino acids gave rise to a reduction in the colour produced by uric acid with the reagents when present in concentrations similar to those found in blood serum. Addition of glycine to serum tungstic acid filtrates in amounts estimated to double the amino acid nitrogen content gave an average reduction of 11·5% in the uric acid assays (three sera tested) as determined by the method of Folin (1933). A serum (J.L.) from a patient with severe gout and renal failure had a uric acid content of 11·2 mg./100 ml. and gave a recovery of added uric acid of 59·5% by this method. The serum had a markedly raised amino acid content.

Table 2. *Recovery of uric acid added to serum determined by three methods*

Uric acid added ranged from 4 to 12 mg./100 ml. of serum.

Method	No. of tests	Average recovery (%)	S.D.
Folin (1933)	19	76·8	4·85
Brown (1945)	11	74·3	5·88
Bidmead (1951)	8	76·5	4·85

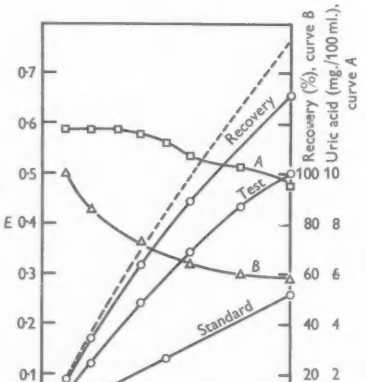

Fig. 4. Optical density produced when different volumes of tungstic acid filtrate alone (Test) and containing added uric acid (5 μg./ml.) (Recovery) are made up to 1 ml. and treated with 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea and 0·5 ml. of phosphotungstate reagent at 35° for 30 min. The broken curve is the algebraic sum of the test and standard (5 μg./ml.) curves. Curve *A* is the calculated serum uric acid and curve *B* the calculated percentage recovery.

Effect of diluting filtrate. Volumes of tungstic acid filtrate prepared from serum both with and without addition of uric acid were diluted with water to 1 ml. and treated with 5% sodium cyanide–15% urea solution (2 ml.) and the phosphotungstate reagent (0·5 ml.). A series of uric acid standards were similarly treated. Fig. 4 shows typical curves from a series of tests. Comparison of the 'standard' with the 'test' curve gives the uric acid concentration of the original serum for the appropriate filtrate dilution (curve *A*), and comparison with the 'recovery' curve gives the percentage recovery of added uric acid for each filtrate dilution (curve *B*). A similar pattern was obtained with urines, assays reaching a constant value when the urine dilution is greater than 1 in 400. These results indicate that recovery of uric acid is improved by using suitable dilutions of tungstic acid filtrate or urine; with serum less than 0·3 ml. of filtrate, and with urine less than 0·25 ml. of a 1 in 100 dilution should be present in 3·5 ml. of reaction mixture.

Modified method. On the basis of the results obtained in the experiments described above, the method of Folin for the determination of uric acid in serum or urine has been modified as follows:

Tungstic acid filtrate (0·2 ml.), 4% (w/v) sodium cyanide containing 15% (w/v) of urea (3 ml.) and Folin's reagent (0·5 ml.) are mixed and heated at 35° for 30 min. Standard solutions should be run alongside the tests. One containing 0·5 mg. of uric acid/100 ml. is usually satisfactory. The tubes are finally cooled and the optical densities of the solutions are read at 690–700 mμ or with a red filter in a photometer either directly or after adjusting to a convenient volume with water. Duplicate tests agree to within 0·12 mg./100 ml. of serum and recoveries are 96·8% (S.D. 6·3) in 25 tests on sera with a range of uric acid contents of 3·3–9·4 mg./100 ml. and added uric acid of from 4 to 10 mg./100 ml. The method can be applied to urine, 0·2 ml. of a 1 in 100 dilution being used for the reaction.

DISCUSSION

Published methods for the determination of uric acid in serum vary in the proportions of the reactants. The experiments described indicate that these proportions may materially affect the results of assays, particularly when the latter are high. The temperature and duration of the reaction have no influence on assays and conditions should be chosen to yield a high $E_{test}:E_{blank}$ ratio.

Sodium cyanide samples which gave different colour yields with a given amount of uric acid were assayed for total cyanide and titratable alkalinity, but little consistent agreement was obtained. One commercial grade giving a low colour yield had a low assay and revealed traces of iron on analysis, the latter in the ferric form considerably reduces the colour yield of uric acid. Changes in alkalinity of the reaction mixture influence considerably the maximum optical density produced by a fixed quantity of uric acid.

Several substances modify the colour reaction; the most significant in the assay of serum are the amino acids. Dilution of the test solutions has been shown to reduce this effect and to yield higher recoveries and subsequent higher assays.

SUMMARY

1. In the reaction between uric acid and phosphotungstic acid in the presence of sodium cyanide, the effects of time, temperature, concentration of reactants and alkalinity of the reaction mixture have been investigated.

2. A method is described which is suitable for the determination of uric acid in as little as 0·05 ml. of serum containing up to 25 mg. of uric acid/100 ml.

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Further Studies on the Cysteine–Cystine Content of the Foetal-Human Haemoglobin

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In a previous paper (Hommes, Santema-Drinkwaard & Huisman, 1956) we described analyses of the total half-cystine content and the number of free sulphydryl groups in foetal-human haemoglobin. With a chromatographic procedure (Schram, Moore & Bigwood, 1954; Kimmel, Thompson & Smith, 1955), a total number of six half-cystine residues was found, whereas with an amperometric-titration method with silver nitrate only four free –SH groups could be demonstrated. These data suggest the existence of one disulphide bridge in the foetal haemoglobin.

In this paper the total number of free –SH groups in this protein are given, as established by two other techniques. In addition, an attempt was made to divide the foetal haemoglobin into two different parts by reduction of the disulphide bridge which should be present. It should thus be possible to get indications if the disulphide linkage is present in one polypeptide chain or between two polypeptide chains. In this study the results obtained with foetal haemoglobin are compared with those for the adult component.

METHODS

Normal adult haemoglobin, obtained from healthy adults (laboratory workers) and pure foetal haemoglobin were prepared as described earlier (van der Schaaf & Huisman, 1955). The carboxyhaemoglobins were stored at 0° for no longer than 1 week. No experimental difference was found between this preparation of adult haemoglobin and that obtained by starch electrophoresis according to Kunkel & Wallenius (1955). Amperometric titrations with $AgNO_3$ and 0·05 M-NH_4NO_3–aq. 0·1 M-NH_3 soln. as supporting electrolyte [Hommes *et al.* (1956) described in detail in the Addendum] followed the method of Benesch, Lardy & Benesch (1955). In this amperometric method a titration mixture of 0·133 M-2-amino-2-hydroxymethylpropane-1:3-diol, 0·1133 M-HNO_3 and 0·01 M-KCl was used in all experiments. The titrimetric experiments were carried out at room temperature. No change was found on lowering the

temperature to 2° (Murayama, 1956, 1957). The results given by these two amperometric techniques were also compared with those obtained with the spectrophotometric method with *p*-chloromercuribenzoate (PCMB) (Boyer, 1954). The increase in optical density caused by the interaction of the reagent with the –SH groups of the haemoglobin (in a 0·2% solution in 0·1 M-acetate, pH 4·6) was measured at 255 mμ. This increase was obtained by subtracting the optical densities of a haemoglobin solution and of a PCMB solution of corresponding concentrations from the optical density of a mixture of haemoglobin and PCMB. The number of –SH groups was estimated from this calculated extinction increment by using 6000 as the molar-extinction increment for the mercaptide formed at 255 mμ (Boyer, 1954). In some experiments denaturing agents such as a polyoxyethylene lauryl alcohol (Brij 35) in a final concentration of 1% and urea (4 M) were used.

For the reduction of the –S–S– bridge, possibly present in the foetal protein, the method of Lindley (1955) was used. Small amounts (3–5 mg.) of protein were incubated for 4 hr. with 0·5 M-lithium thioglycollate soln. After addition of a mixture of 39 ml. of acetone and 1 ml. of N-HCl, the precipitate (consisting only of the globin part of the haemoglobin) was removed by centrifuging. This precipitate was treated as for other proteins (Lindley, 1955) by coupling it with iodoacetamide, and was finally dissolved in a small amount of 0·1 M-thioglycollic acid soln. This protein solution was submitted to paper electrophoresis (Whatman no. 3 MM) for 16 hr. with an 0·1 M-buffer solution of lithium thioglycollate, pH 5·1, with addition of 4 M-urea (300 v at 10 mA). Colour development was obtained with ninhydrin after drying at 120° and washing the paper with ethanol to remove the urea. Larger amounts of this protein solution were prepared and investigated by moving-boundary electrophoresis with the same buffer solution. Control experiments with globin solutions were carried out, 0·1 M-glycine buffer, pH 5·1, with addition of 4 M-urea, being used.

RESULTS

The results obtained with the two amperometric-titration methods and the spectrophotometric method (PCMB method) are given in Table 1. These analyses were carried out both for normal adult haemoglobin and the foetal pigment. The data are compared with those reported in an earlier paper (Hommes *et al.* 1956). First the results obtained with the amperometric-titration method, with NH_4NO_3–aq. NH_3 soln. as supporting electrolyte, were confirmed. For both haemoglobins the results with the method of Benesch *et al.* (1955) are 10–20% higher than those with the first titration method, which is in accordance with the data given by Benesch *et al.* (1955). No clear explanation can be given for this phenomenon. It may be due to the formation of complexes of protein–silver mercaptides with additional Ag^+ ions, which are less stable after denaturation. Addition of a high concentration of urea decreases the number of titratable –SH groups. The values obtained with the tris method, in 4 M-urea, are identical with those found with the NH_4NO_3–aq. NH_3 soln. method. The data obtained with the PCMB method are in poor agreement. The total numbers of –SH groups for adult and foetal haemoglobins were found to be 6·7 and 2·8 respectively, whereas amperometric-titration methods gave 7·6 and 4·1. Addition of a denaturing agent (1% of Brij 35) resulted in a decrease of the reactive –SH groups. The difference between the results obtained with the PCMB method and with the amperometric techniques may be due to the choice of a molar-absorption increment of 6000. According to Boyer (1954) the molar-absorption increment is not identical for mercaptides in different proteins. The method is therefore suitable mainly for comparative studies. A similarly lower amount of reactive –SH groups was found to be present in the foetal haemoglobin compared with the adult component as estimated with both amperometric-titration techniques.

In order to prove the hypothesis of the existence of a disulphide bridge in the foetal haemoglobin more directly some investigations were carried out by reducing this linkage with thioglycollic acid according to the method of Lindley (1955). The reduced protein was studied, after coupling of the

Table 1. *Sulphydryl content of normal adult and foetal-human haemoglobin*

Values are given as half-cystine residues/mole of haemoglobin, mol.wt. 68 000. *n* = Number of determinations.

	Haemoglobin					
	Adult			Foetal		
Method	*n*	Found	Assumed	*n*	Found	Assumed
Chemical	15	8·28±0·17	8	15	5·67±0·15	6
Amperometric titration*	10	7·6±0·10	8	10	4·0±0·10	4
Amperometric titration*	5	7·5±0·06	8	12	4·1±0·08	4
Benesch *et al.* (1955)†	2	9·6	—	3	4·4	—
Benesch *et al.* (1955)† + 4 M-urea	2	7·6	8	3	4·1	4
PCMB	10	6·7±0·3	—	10	2·8±0·4	—
PCMB + 1% Brij 35	10	2·7	—	10	2·4	—

* Supporting electrolyte: 0·05 M-NH_4NO_3–aq. 0·1 M-NH_3 soln.
† Supporting electrolyte: 0·133 M-tris–0·1133 M-HNO_3–0·01 M-KCl.

free –SH groups with iodoacetamide, both by paper electrophoresis and by the moving-boundary technique. If a disulphide linkage were present between two polypeptide chains two different components might be formed, which could be separated by these techniques. The paper-electrophoretic patterns of the adult and foetal haemoglobins, given in Fig. 1, show two well-marked spots (in addition to a small amount at the starting-point) with foetal haemoglobin, whereas only one spot was found for the adult haemoglobin.

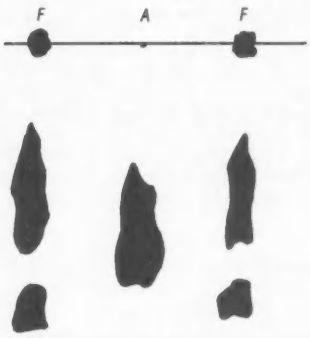

Fig. 1. Paper-electrophoretic pattern of adult (*A*) and foetal (*F*) haemoglobin after reduction with thioglycollic acid and coupling with iodoacetamide. Whatman no. 3MM; 0·1 M-lithium thioglycollate, pH 5·1, with addition of 4 M-urea.

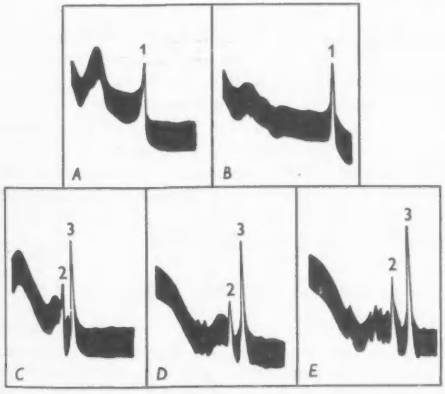

Fig. 2. Electrophoretic pattern of adult and foetal haemoglobins after reduction with thioglycollic acid and coupling with iodoacetamide. Buffer solution: 0·1 M-lithium thioglycollate, pH 5·1, with 4 M-urea (300 v at 10 mA). (*A*) and (*B*) normal adult haemoglobin: (*A*) after 2½ hr.; (*B*) after 4¼ hr. (*C*), (*D*) and (*E*) foetal haemoglobin: (*C*) after 3 hr.; (*D*) after 4½ hr.; (*E*) after 5 hr.

Similar results were obtained with moving-boundary electrophoresis. Fig. 2 shows only one component (marked 1) for the adult haemoglobin, whereas two distinct components (marked 2 and 3) are present with the foetal haemoglobin. Without reduction with thioglycollic acid no splitting of the foetal component occurred (Fig. 3) when this protein was studied by the moving-boundary electrophoresis with a glycine-buffer solution of the same pH (5·1) and with the same high concentration of urea (4 M).

DISCUSSION

The results of the different methods for the estimation of the total numbers of free –SH groups in the foetal haemoglobin support the previous finding (Hommes *et al.* 1956) that only four of the six half-cystine residues present in foetal haemoglobin are reactive sulphydryl groups. These analyses suggest the presence of a disulphide bridge in this protein. As all the eight half-cystine residues in the normal adult haemoglobin are found to be present as free –SH groups no indication of such a linkage in this protein was obtained. The electrophoretic studies show that the foetal haemoglobin is divided into two different components by a reducing agent, probably by reduction of a disulphide bridge. It is therefore likely that the disulphide linkage is present between two polypeptide chains rather than in one polypeptide chain only. These results and the findings that only two valyl residues are present in *N*-terminal position (Porter & Sanger, 1948 and Huisman & Drinkwaard, 1955), and probably one histidine and one tyrosine in *C*-terminal position (Huisman & Dozy, 1956), support the hypothesis (Hommes *et al.* 1956) that foetal haemoglobin is built up by two large and different polypeptide chains, connected at least to each other by one disulphide bridge. Further studies,

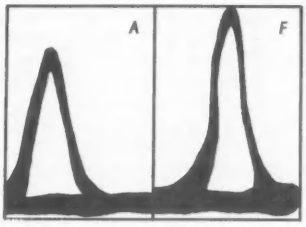

Fig. 3. Electrophoretic pattern of the globins of adult (*A*) and foetal (*F*) haemoglobins with 0·1 M-glycine buffer soln., pH 5·1, with addition of 4 M-urea.

such as a more direct investigation of the chemical characteristics (*N*- and *C*-terminal residues) of the two different parts, will be valuable in confirming this structure of the foetal haemoglobin.

SUMMARY

1. Previous reports that only four of the total number of six half-cystine residues present in foetal-human haemoglobin are reactive sulphydryl groups were confirmed by two other techniques.

2. After reduction with thioglycollic acid reduced foetal haemoglobin was studied by paper and moving-boundary electrophoresis. With both techniques two different components were found.

3. These results support the hypothesis that foetal-human haemoglobin is built up of two different polypeptide chains connected at least to each other by one disulphide bridge.

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ADDENDUM

Amperometric Titration of Sulphydryl Groups in Human Haemoglobin with Silver Nitrate

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The slightly different amperometric-titration methods with silver, used for the estimation of sulphydryl groups in haemoglobins, have not always given identical results. Ingbar & Kass (1951), for instance, employing a titration medium of 0·85% NaCl found 2 moles of –SH/mole of normal adult haemoglobin and 3 moles of –SH/mole of sickle-cell haemoglobin. Addition of 20% methanol increases this number to 4–5 moles/mole of haemoglobin. Benesch, Lardy & Benesch (1955), using a 2-amino-2-hydroxymethylpropane-1:3-diol (tris) solution as supporting medium, obtained 9·6 moles of –SH/mole of native normal adult haemoglobin, whereas after denaturation with 8 M-urea this number was reduced to 8 moles of –SH/mole of adult haemoglobin.

With NH_4NO_3–aq. NH_3 soln. buffers Ingram (1955) has found that the number of titratable –SH groups in this protein decreases with increasing concentration of ammonia. He concluded that there are 4 moles of –SH/mole of native adult human haemoglobin, a number which increased to 8 after denaturation with sodium dodecyl sulphate. In previous papers (Hommes, Santema-Drinkwaard & Huisman, 1956; Hommes, Dozy & Huisman, 1958) we reported the presence of 8 –SH groups/molecule of native adult human haemoglobin. The method used was similar to that of Ingram (1955) except that it was found necessary to pretreat the electrode by immersing it in an ammoniacal $HgCl_2$ solution. As Ingram has recently (1957) described different results obtained with this modified method, it is important to describe our technique in fuller detail.

METHODS AND RESULTS

The haemoglobin solutions were prepared as described earlier (van der Schaaf & Huisman, 1955). Washed red-blood cells were haemolysed by the addition of 1 vol. of water and 0·4 vol. of toluene. This mixture was stored for 24 hr. at 0°. After centrifuging and removal of the toluene layer the haemoglobin solution was shaken for 1 min. with Celite 535 (1 g./100 ml. of haemoglobin soln.) and slowly filtered with suction on a Büchner funnel. The clear haemoglobin solution was finally saturated with carbon monoxide and dialysed

against twice-distilled water for 48 hr. at 4° with eight changes of the dialysing fluid. A haemoglobin solution prepared in this way showed no denaturation of the protein, as measured by electrophoretic, chromatographic and salting-out experiments (Huisman & Schreuder, 1957).

The argentometric titration was first performed in 50 ml. of solution with a dropping-mercury electrode. The solution contained 0·05 M-NH_4NO_3 and different concentrations of aq. NH_3 soln. (0·02 M, 0·10 M and 0·25 M), with $0·4–0·7 \times 10^{-5}$ mole of carboxyhaemoglobin. The haemoglobin concentration was estimated spectrophotometrically as cyanmethaemoglobin at 540 and 544 mμ. The molar-extinction coefficient at both wavelengths is $1·15 \times 10^4$, calculated on a molecular weight of 16 700.

The titrations were carried out with a 20 mM-$AgNO_3$ solution (Merck and Co. Ltd). The diffusion current to the dropping-mercury electrode was measured with a Kipp galvanometer (A 70). A potential of −0·3 v was applied. The results of the titration of the normal adult haemoglobin are given in Table 1.

Table 1. *Sulphydryl content of normal adult human haemoglobin estimated by the argentometric-titration method with a dropping-mercury electrode*

Concn. of NH_3 (M)	Number of titrations	Moles of –SH/mole of adult haemoglobin
0·02	7	8·7±0·2
0·10	7	8·7±0·2
0·25	7	8·5±0·2

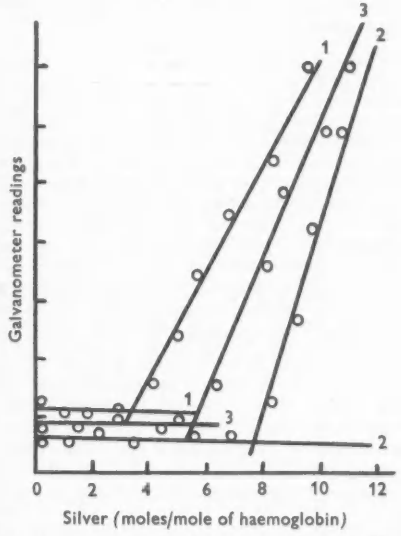

Fig. 1. Argentometric-titration curves of adult human haemoglobin under different conditions (for explanation see text).

Argentometric titrations were next undertaken with an untreated, clean, rotating platinum-wire electrode (900 rev./min.). The supporting electrolyte contained 0·05 M-NH_4NO_3–aq. 0·1 M-NH_3 soln., and the volume in the titration vessel was again 50 ml., containing the same amount of 0·4–$0·7 \times 10^{-5}$ mole of carboxyhaemoglobin. The same potential of −0·3 v was applied. The number of titratable –SH groups/mole of haemoglobin was now found to be 3·6 ± 0·15 (eight titrations). Pretreatment of the platinum wire with an ammoniacal $HgCl_2$ solution (30–45 min. at −0·2 v versus a standard calomel electrode) resulted in an increase of the titratable –SH groups to 7·6 ± 0·1 (ten titrations). Fig. 1 shows some titration curves without pretreatment (curve 1) and after pretreatment of the platinum electrode with the ammoniacal $HgCl_2$ solution (curve 2). Repetition of the titration experiments with the treated electrode, but without repeating the pretreatment of the platinum wire, resulted in a gradual shift of the titration curves to the left (for instance, Fig. 1, curve 3) and therefore to a smaller number of titratable –SH groups. Finally, a value of 3·6 –SH groups/mole of haemoglobin was reached (Fig. 1, curve 1), which did not change in further titration experiments. It will be noted that in these titration curves only one sharp end-point was obtained.

DISCUSSION

Ingram (1955) described a marked influence of the ammonia concentration with an untreated rotating platinum-wire electrode. With the dropping-mercury electrode, however, no distinct effect of ammonia concentration was found (Table 1).

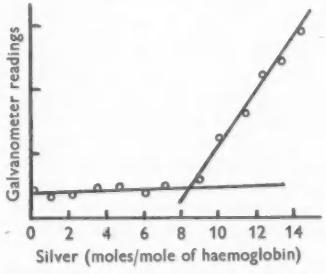

Fig. 2. Argentometric-titration curve of adult human haemoglobin with the dropping-mercury electrode and 0·05 M-NH_4NO_3–aq. 0·1 M-NH_3 soln. as supporting electrolyte.

Although the end-point of the titration curve (Fig. 2) is not very sharp, it offers no explanation for this difference. It seems likely that the ammonia effect has to be explained by the difference in the electrode used. In our comparative studies on the determination of –SH groups in haemoglobins we therefore used a median ammonia concentration (0·1 M) for both untreated and treated platinum-wire electrodes.

An important finding is the effect of the pretreatment of the platinum electrode with ammoniacal mercuric chloride solution before use. The final results obtained with such a system for studying native human haemoglobin are in agreement with the data obtained by Ingram (1957), except that only one end-point was found in our experiments. The differences between the results in titrations with the untreated platinum electrode and with the mercury-coated electrode must be explained rather by the different experimental conditions than by a different reactivity of some of the sulphydryl groups in the haemoglobin, as examination of the current–voltage curves has shown an increased sensitivity of the mercury-coated electrode. Moreover, the results of repeated titrations with a mercury-coated platinum electrode show a gradual decrease in the amount of silver bound. We therefore believe that the number of reactive –SH groups of different haemoglobins, and perhaps of other proteins, can be studied in the native state and after denaturation very clearly by the procedure using a mercury-coated platinum-wire electrode.

SUMMARY

1. A detailed description of the argentometric-titration procedure with a rotating mercury-coated platinum electrode for the estimation of the number of reactive sulphydryl groups in normal adult human haemoglobin is given.

2. A comparison of this method with the technique using an untreated platinum electrode revealed that with the latter procedure lower amounts of titratable –SH groups were found to be present in native adult human haemoglobin. It is suggested that this decrease is caused by the different experimental conditions.

3. The titration curves obtained by amperometric titration of native normal adult human haemoglobin with silver nitrate at the mercury-treated platinum electrode show only one sharp end-point.

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The Determination of Phylloerythrin in Blood

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Diseases of livestock in which there is an acute sensitivity of exposed parts to sunlight occur in many parts of the world. The need to identify the photodynamic agents responsible has been stressed (Clare, 1952). Photosensitivity directly due to components of the diet has been shown to occur in some instances, such as in poisoning by plants of the genus *Hypericum* (Pace, 1942), by *Fagopyrum esculentum* (Wender, Gortner & Inman, 1943), and after administration of phenothiazine to calves (Clare, 1947) and lambs (Gordon & Green, 1951).

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More commonly, where liver damage is also found, the pigment phylloerythrin may be responsible (Clare, 1952). Phylloerythrin has been shown conclusively to be the photodynamic agent in geeldikkop (Rimington & Quin, 1934), in facial eczema (Clare, 1944), in congenital photosensitivity in Southdown sheep (Clare, 1945), in bile-duct ligation in sheep (Rimington & Quin, 1934) and in poisoning of sheep by *Lippia rehmanni*, *Panicum miliaceum*, *Myoporum laetum* and copper (Clare, 1952).

Phylloerythrin, a normal component of the bile and faeces of herbivores, is formed by microbial degradation of chlorophyll and related pigments in

their passage through the gut (Quin, Rimington & Roets, 1935), from which it is partially absorbed and re-excreted into the bile. In certain types of liver dysfunction, phylloerythrin accumulates in the peripheral circulation, causing intense photosensitization.

In photosensitivity diseases where the photodynamic agent is unknown it is desirable to examine affected animals for phylloerythrinaemia, but no quantitative method for the estimation of phylloerythrin in blood or urine has previously been described.

To provide information on levels of blood phylloerythrin in photosensitive animals, and as part of an investigation of sporadic photosensitization in sheep and cattle, a method has been developed for the estimation of phylloerythrin in small blood samples. This method depends on the measurement of the light absorption of phylloerythrin in ether at one or other of the peaks in the banded absorption spectrum in the visible and the near ultraviolet.

The procedure described in this paper is based on Fischer's (1926) method of porphyrin extraction from blood serum, modified slightly to obtain a more granular protein precipitate. The number of extractions, volumes and concentrations specified was selected to minimize interference by protoporphyrin without undue loss of phylloerythrin, and were checked with normal sheep plasma to which had been added known amounts of phylloerythrin and protoporphyrin.

EXPERIMENTAL

Spectrophotometry. All measurements were made on a Beckman model DU photoelectric quartz spectrophotometer checked frequently for wavelength calibration against the more intense lines of a mercury arc between 2225 and 5791 Å. For phylloerythrin estimations the slit width was set at 0·03 mm. (band width 7 Å at 4200 Å). The microcells (Pyrocell Manufacturing Co., U.S.A.) had a minimum volume of 100 mm.^{3} and a light path of 1 cm.

Reagents. All reagents except ethyl ether were A.R. grade. Ether was laboratory-reagent grade purified by shaking with acid $FeSO_4$ solution and redistilling.

Pure phylloerythrin was prepared from dried ox bile by the method of Rimington & Quin (1934). The ether extract was washed with 2% (w/v) HCl during repeated transferences between ether and 10% (w/v) HCl. The 10% HCl solution was extracted with chloroform, which was washed with water, dried and evaporated in air. The dried material was taken up in a small volume of pyridine and the cycle repeated several times. After two such cycles no changes were observed in the number and positions of the absorption maxima or in their relative intensity. The final chloroform extract was slowly evaporated, yielding a crystalline material that was used without further purification. The visible absorption maxima in ether (519·5, 560·0, 584·0, 638·0 mμ) are in reasonable agreement with earlier data for phylloerythrin in this solvent [e.g. Hellström (1931): 512·3–526·6, 558·0, 579·8, 595·0, 635·2 mμ; Fischer (1933): 521, 561, 584, 593, 637 mμ; Keys & Brugsch (1938): 521, 559, 588, 637 mμ; Clare (1944): 522, 561, 585–597, 636 mμ]. These maxima and the ratios of their intensities (1·0, 1·52, 1·03, 0·220 respectively) agree closely with values obtained for phylloerythrin in dioxan by Stern & Wenderlein (1935) (517·5, 557·5, 581·5, 634 mμ; intensities 1·0, 1·52, 1·00, 0·218) and Lemberg & Falk (1951) (519, 559, 583·5, 637·5 mμ; intensities 1·0, 1·47, 0·98, 0·224).

Protoporphyrin was prepared from haemoglobin by the method of Keys & Brugsch (1938) and purified by repeated distribution between ethyl ether and aq. HCl solutions. Absorption maxima in ether–acetic acid were at 5030, 5365, 5780 and 6320 Å, in good agreement with the values of 5020, 5370, 5760 and 6325 Å given by Lemberg & Legge (1949*a*). The concentration of protoporphyrin in the final ether solution was determined by the method of Grinstein & Wintrobe (1948).

Procedure. Serum or plasma (10 ml.) was shaken with ethyl ether (20 ml.), followed by the addition of ether–acetic acid (1:1; 30 ml.). The flask was stoppered, shaken and left for 30 min. The ether layer was decanted into a separating funnel and the residue washed with ether (3 × 10 ml.), which was allowed to stand in contact for several minutes before decanting. The combined extracts were shaken with 2% (w/v, 0·55 M) HCl (3 × 20 ml.). The combined HCl washings were extracted with ether (2 × 5 ml.), the ether layers being transferred by teat pipette to the original ether extract, which was then shaken with 20% (w/v, 6 M) HCl (3 × 4 ml.). The combined 20% HCl extracts were centrifuged to ensure separation of any ether emulsion and washed with ethyl ether (1–2 ml.) to remove traces of ether-soluble pigments. Ether (2 ml.) was added before shaking with alkaline sodium acetate (25 ml. of 0·9 M-sodium acetate; 1·5 M in NaOH), followed by further extraction with ether (3 × 3 ml.). This amount of alkaline sodium acetate left the aqueous phase slightly acid and thereby reduced further the risk of contamination by porphyrins of lower acid number. The ether extracts were mixed in a stoppered graduated cylinder and the volume was noted.

The optical density of the ether solution was measured with stoppered 2 cm. cells at 4120, 4140, 4170, 4230, 4290 and 4700 Å, against water. The phylloerythrin concentration in the original plasma was obtained from the relation

$$c\ (\text{mg./100 ml.}) = 0{\cdot}020\,V\,(E_{4140} - E_{4700}),$$

where V is the final volume of ether. The factor 0·020 includes a correction for the recovery of only 87% of phylloerythrin (see below).

The reliability of the determination was checked:

(i) by comparing $(E_{4170} - E_{4230}) - (E_{4230} - E_{4290})$ with $(E_{4140} - E_{4700})$. The ratio, R, of these quantities should be close to 0·23;

(ii) by confirming that E_{4140} was greater than E_{4120} and E_{4170}.

For more dilute solutions approximate values can be obtained by extracting the final ether solution into 20% HCl (1 ml.) and driving any phylloerythrin back into a small volume of ether with alkaline sodium acetate (2 ml.).

Higher concentrations can be measured with shorter absorption cells, dilution of the ether extract or absorption measurements in the visible. Beer's Law is obeyed at the four visible maxima for concentrations of phylloerythrin in

ether up to at least 3·5 mg./100 ml., and the ratios of the optical densities at the maxima provide a purity check. The phylloerythrin in the original plasma is given by

$$c\ (\text{mg./100 ml.}) = 0{\cdot}23\ VE_{5600}.$$

RESULTS

Absorption spectrum of phylloerythrin

Fig. 1 shows the absorption spectrum of phylloerythrin in ethyl ether saturated with aq. acetic acid under the conditions described above for the estimation of phylloerythrin. Observed maxima, minima and extinction coefficients are given in Table 1.

The shape of the absorption curve and the positions of the maxima were unchanged when the ether extract was washed with water and with 2% HCl.

The inflexion at 4260 Å, which is absent from the spectral-absorption curves for most porphyrins, including protoporphyrin, mesoporphyrin and coproporphyrin, provides a convenient check on the purity of solutions prepared for phylloerythrin estimation. Over the successive and equal wavelength intervals 4170–4230, 4230–4290 Å, there is a considerable difference in the optical-density increments (Fig. 2). This difference

$$[d = (E_{4170} - E_{4230}) - (E_{4230} - E_{4290})]$$

is a constant fraction of the optical densities at any selected wavelengths, e.g. 4140 and 4700 Å. This follows directly from Beer's Law ($E = \epsilon ct$), which holds for all wavelengths. Thus

$$d = [(\epsilon_{4170} - \epsilon_{4230}) - (\epsilon_{4230} - \epsilon_{4290})]\ ct,$$

and

$$E_{4140} - E_{4700} = (\epsilon_{4140} - \epsilon_{4700})\ ct.$$

Hence

$$d = \frac{(\epsilon_{4170} - \epsilon_{4230}) - (\epsilon_{4230} - \epsilon_{4290})}{(\epsilon_{4140} - \epsilon_{4700})}\ (E_{4140} - E_{4700})$$

$$= R\ (E_{4140} - E_{4700}),$$

where R is a constant.

For phylloerythrin in ether R has a value of 0·23. The inflexion is absent from solutions of phylloerythrin in aqueous hydrochloric acid. Although the inflexion is also absent from solutions of protoporphyrin in ether the absorption spectrum of this pigment shows sufficient curvature between 4170 and 4290 Å to give a value of $R = 0{\cdot}10$.

Sensitivity

The method as described enables plasma phylloerythrin levels to be determined over the range 0·05–0·5 mg./100 ml. The use of visible absorption-spectrum measurements for high plasma phylloerythrin concentrations extends this range to

Fig. 1. Absorption spectrum of phylloerythrin in ethyl ether saturated with aq. acetic acid.

Table 1. *Phylloerythrin in ether–acetic acid*

Maxima (Å)	Minima (Å)	$E^{1\%}_{1\,cm.}$
4140	—	2800
4260 (inflexion)	—	1560
—	4760 (very flat)	50
5195	—	159
—	5420	82
5600	—	242
—	5735	99
5840	—	163
—	6215	13·3
6380	—	35

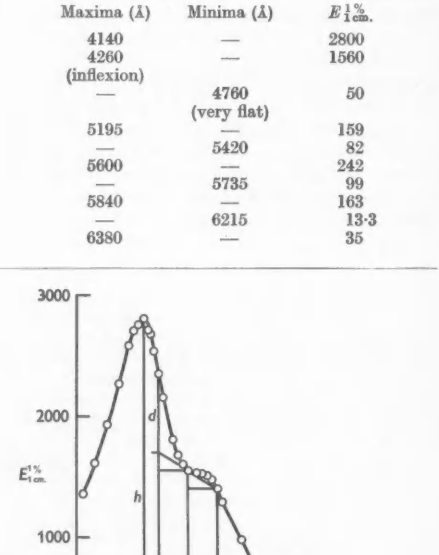

Fig. 2. Use of inflexion in absorption spectrum of phylloerythrin in ether saturated with aq. acetic acid as purity check. The ratio $d:h$ (0·23) is independent of concentration.

3·5 mg./100 ml. By the use of microcells and application of the R test in the modified method for very low levels of phylloerythrin a limit of detection of the order of 0·005 mg./100 ml. is given.

Specificity

The wavelength of the Soret maximum provides a useful qualitative check on the pigment obtained in the method described. Thus phylloerythrin in ether has $\lambda_{max.} = 4140$ Å; protoporphyrin, $\lambda_{max.} = 4040$ Å. Owing to the sharpness of the Soret band and the rapid rate of change of optical density with wavelength near the maximum, the position of the maximum is relatively little affected by the presence in the solution of compounds such as bilirubin, which show a more general light-absorption. For this reason an independent check on the purity of the ether extracts is necessary and this is provided, quantitatively, by calculation of R.

The hydrochloric acid number of protoporphyrin [2·0, Keys & Brugsch (1938)] is close enough to that of phylloerythrin [8·5, Keys & Brugsch (1938)] for significant amounts to be carried through. In human red-blood cells normal protoporphyrin levels are less than 0·05 mg./100 ml. (Cartwright, Lauritsen, Jones, Merrill & Wintrobe, 1946); in sheep the values are 0·03–0·09 (Allen, 1956). Kench, Gillam & Lane (1942) found whole-blood protoporphyrin concentrations up to 0·4 mg./100 ml. in human chronic lead-poisoning cases and reported that in this condition generally protoporphyrin concentrations were higher in plasma than in red cells. Values up to 1 mg./100 ml. of red blood cells have been reported (Dr E. C. Vigliani, quoted by Lemberg & Legge, 1949*b*). Recovery experiments with protoporphyrin added to plasma showed that a plasma protoporphyrin concentration of 1 mg./100 ml. gave an apparent phylloerythrin value of 0·04 mg./100 ml. This was shown to be spurious when R was found to be 0·10 and not 0·23 as required for phylloerythrin. Concentrations of protoporphyrin high enough to cause significant interference are unlikely to be encountered in practice.

Deutero-, meso-, haemato- and copro-porphyrins, which are known or believed to occur in normal or pathological metabolism, have much lower hydrochloric acid numbers (Keys & Brugsch, 1938), and present no difficulty in obtaining quantitative separation from phylloerythrin. Uroporphyrin is insoluble in ether acidified with acetic acid.

Bilirubin, biliverdin and carotene (which is a normal constituent of bovine plasma) are quantitatively rejected in the extraction process described. Haemolysis of the sample does not affect the determination.

The possible effects of chlorophyll-degradation products other than phylloerythrin have not been investigated experimentally. However, Rothemund (1935), in his examination of ox bile for porphyrins derived from chlorophyll, found only phylloerythrin and smaller quantities of pyrroporphyrin, and no phylloporphyrin or rhodoporphyrin. Pyrroporphyrin, with an acid number of 1·5, is unlikely to interfere in the estimation of phylloerythrin by the method described.

Recoveries. The extraction process entails some loss of phylloerythrin because of the necessity to remove most of the protoporphyrin, the greatest loss occurring during the washing with 2% hydrochloric acid. The extent of the overall loss was determined by recovery experiments. Recoveries of phylloerythrin in ether added to plasma, within the range 0·06–1·37 mg./100 ml., followed by evaporation of the ether, amounted to 87 ± 6% (range 80–96). On the basis of these results, multiplication by 1·15 is included in the factor used to convert optical densities into plasma phylloerythrin concentrations.

Below about 0·05 mg./100 ml. differences are not sufficiently great for accurate calculation of R and hence there is no reliable check on the apparent phylloerythrin values obtained. In a series of experiments with plasma to which had been added 0·007–0·032 mg. of phylloerythrin/100 ml., recoveries were 0·012–0·054 mg./100 ml., including a (spurious) plasma blank of 0·004 mg./100 ml. In a typical experiment an ether extract with an apparent phylloerythrin content of 0·015 mg./100 ml. was concentrated into 0·5 ml. of ether. Measurement by microcell (slit width 0·06 mm.) gave the original phylloerythrin level as 0·007 mg./100 ml., $R = 0·19$.

DISCUSSION

In their work on geeldikkop, Rimington & Quin (1934) isolated phylloerythrin from large volumes of blood from affected sheep but did not determine its concentration. Clare (1944), using existing methods of porphyrin extraction and various modifications, followed by fluorimetric estimation, was unable to obtain even roughly quantitative recoveries of phylloerythrin added to blood, and in some of his experimental sheep photosenisitization after injection of phylloerythrin occurred when peripheral-blood phylloerythrin had fallen below levels he could detect. Clare concluded that if, in affected sheep, the pigment was found in the blood sufficient was present to cause photosensitivity.

The present method enables phylloerythrin in low concentrations in blood to be identified and estimated. The use of an internal spectroscopic check which depends on the shape of the phylloerythrin-absorption curve considerably increases the reliability of the determination. The sharpness

of the phylloerythrin absorption bands and the dependence of R on small wavelength differences require accurate calibration of the spectrophotometer and the use of narrow slit widths.

The highest plasma phylloerythrin level so far found with the method described has been 0·3 mg./100 ml., in a sheep poisoned by *Panicum miliaceum*, although Clare (1944) estimates that levels up to at least 1·6 mg./100 ml. occur in severe cases of facial eczema in sheep.

The method appears to be applicable to other biological materials, especially urine. Phylloerythrin has been detected in the urine of humans on vegetable diets (Kemeri, 1924; Fischer & Hilmer, 1925), indicating that absorption of chlorophyll-degradation products from the gut can take place in humans. Whether phylloerythrinaemia occurs in human-liver dysfunctions and porphyrias is not at present known, but the method described makes feasible the examination of blood from affected persons. In addition to its application to photosensitivities of unknown origin it also enables information to be obtained on the concentrations of phylloerythrin involved in photosensitization in sheep and cattle.

SUMMARY

1. Details are given of a method for estimating phylloerythrin in blood, consisting in extraction into ether–acetic acid solution, distribution between ether and hydrochloric acid solutions, and spectrophotometric measurement.

2. The inflexion at 4260 Å in the absorption spectrum of phylloerythrin provides a quantitative check on the purity of the material being measured.

3. The method is applicable over the range 0·05–3·5 mg. of phylloerythrin/100 ml. of plasma, but a modification giving approximate values down to 0·005 mg./100 ml. is described. Protoporphyrin and other porphyrins do not cause significant interference.

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APPENDIX

The Form in which Phylloerythrin Occurs in Bile and Blood

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Although phylloerythrin is extremely insoluble in water it occurs as a normal constituent of sheep and ox bile, from which it is not thrown down on centrifuging. Similarly, although it can be quantitatively extracted into ether from bile acidified with acetic acid, phylloerythrin is not readily extracted from fresh bile with ether alone. A similar observation was made with plasma from a congenitally photosensitive Southdown sheep. Dialysis of bile in collodion tubes led to the deposition of most of the phylloerythrin on the walls, with very little present in the diffusate. The effect of pH on

extractability of phylloerythrin from bile was tested by adding fresh ox bile to equal volumes of buffer solutions and shaking with ether. In solutions below pH 5 almost all the phylloerythrin was extracted into the ether. Above pH 7·5 less than 5% of the phylloerythrin was present in the ether phase. Between pH 5 and 7·5 intermediate values were obtained. This behaviour could be imitated by adding phylloerythrin (dissolved in a small volume of dilute hydrochloric acid) to solutions of sodium tauroglycocholate, and similar results were also obtained with strong soap solutions. On the other hand, with phosphate buffer solutions of pH 7·5 distribution of phylloerythrin was strongly in favour of the ether phase. As soaps and bile salts in aqueous solution are colloidal electrolytes (Roepke & Mason, 1940) it is concluded that phylloerythrin is present in bile in the free state and is probably maintained in solution by adsorption on the colloidal micelles.

Results from the study of bile suggest that in blood phylloerythrin is dispersed on colloidal micelles such as the bile salts and the plasma proteins. In support of this hypothesis is the observation (Rimington & Quin, 1934) that in sheep affected with geeldikkop phylloerythrin was present entirely in the plasma, only traces being found in the well-washed corpuscles.

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Studies on the Structure of Brain Ganglioside

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The brain gangliosides are macromolecular glycolipids first described by Klenk (1942). They are known to contain neuraminic acid, sphingosine, fatty acids, hexose and hexosamine, although little is known of the manner in which these constituents are combined. The present study reports detailed structural evidence obtained by stepwise hydrolytic cleavage of a highly purified preparation of brain ganglioside leading to the isolation and characterization of a constituent glucocerebroside, and the formulation of the structure of the repeating unit.

METHODS

Nitrogen. The digestion (Gunning, 1889; Arnold & Wedemeyer, 1892) was continued for 45 min. after the solution had become colourless. Water (14 ml.) and one drop of 1% gum ghatti were then added. After 6 min., 8 ml. of Nessler's reagent (Koch & McMeekin, 1924) was added to successive tubes at 45 sec. intervals, the colour allowed to develop for 9 min. and then read at 435 mμ (M. Lees, unpublished work).

Phosphorus. Phosphorus was determined spectrophotometrically (Sperry, 1942).

Neuraminic acid. The humin reaction (Folch, Arsove & Meath, 1951), the direct Ehrlich's reaction (Odin & Werner, 1952) and Bial's orcinol reaction (Bohm, Dauber & Baumeister, 1954) were employed. The Bial's reaction was found to be the most reliable.

Reducing sugar. The methods of Hagedorn & Jensen (1922) and of Hanes (1929) were adapted. Results were expressed as equivalents of galactose or glucose.

Hexose. Hexose was estimated by a modified orcinol–H_2SO_4 method (Sorensen & Haugaard, 1933) and expressed as equivalents of glucose or galactose. Since the absolute values differed slightly for the two sugars, identification of the hexose by paper chromatography indicated which curve was to be used. When glucose and galactose were together in a single fraction, the hexose was expressed as equivalents of galactose, since galactose was always in two- to three-fold excess.

Determination of hexosamine. The following modifications of the Elson & Morgan (1934) reaction were employed: 1 ml. of acetylacetone (6% in 2N-carbonate buffer) was added to a 2 ml. aqueous sample. The covered tube was heated at 100° for 20 min. Colour was developed with Ehrlich's reagent (2·6%) for 45 min. and read at 530 mμ. The standard curve, based on a purified sample of galactosamine hydrochloride, was verified for each determination. This modified method was suitable for estimation of 5–40 μg. of galactosamine (maximum error 3%). Maximum values were obtained after the subfractions were hydrolysed with N-HCl in a sealed tube for 16 hr.

Identification of the hexosamine. The method of Stoffyn & Jeanloz (1954), Gardell, Heijkenskjold & Roch-Norlund (1950) was used. The reaction with ninhydrin was carried out in a sealed capillary tube. The solvent system was modified (see chromatography methods). The amino sugar fractions used were first freed from neutral sugars and other contaminants by chromatography on a column of Dowex 50.

Amino nitrogen. Primary amino nitrogen was determined by the method of Van Slyke (1929).

Paper chromatography. Whatman no. 1 or no. 54 paper was irrigated downwards with *n*-butanol–ethanol–water (4:2:1, by vol.) for 12–72 hr., depending on the separation desired. Silver nitrate was used to develop reducing substances (Trevelyan, Procter & Harrison, 1950), ninhydrin for free amino groups and Ehrlich's reagent for neuraminic acid.

Ion-exchange fractionation. Dowex 50 resin (Boas, 1953) was used to separate neutral from amino sugars. Water was used to elute the neutral sugars, and 2N-HCl to elute the amino sugars.

Dialysis. Washed cellophan was used. In exhaustive dialysis, the outside water was changed at 12 hr. intervals until no solids appeared in the diffusate.

Preparation of brain ganglioside. Hot methanol extraction of whole ox cerebral hemispheres, freed from investing meninges and blood vessels, was employed, followed by precipitation in the cold, and partition between the two phases formed by $CHCl_3$–methanol (2:1, v/v) and water (12·2%, by vol.) (Folch, Meath & Bogoch, 1956; Bogoch, 1956). The upper-phase material, freed from solvents and dialysed, was crude brain ganglioside (P, 0·8%; yield, 2 g./kg. of fresh cerebral hemispheres).

Crude ganglioside was purified by repeated treatment in $CHCl_3$–methanol (2:1, v/v) and water (6·6%, by vol.). (Final yield, 1 g./kg. of whole cerebral hemispheres.) The ash content was reduced from 11% to less than 1·1% by repeated acidification and dialysis in the cold (Folch *et al.* 1951). The final preparation was completely water-soluble, free of phospholipids (P, 0·07%) and of dialysable contaminants. It contained N 2·9%, hexose 24% (expressed as galactose), neuraminic acid 30·3% and free amino N 0·5%. The preparation was homogeneous by electrophoresis, and over 95% homogeneous in the ultracentrifuge. Minimum molecular weight, 250 000 (Bogoch, 1956).

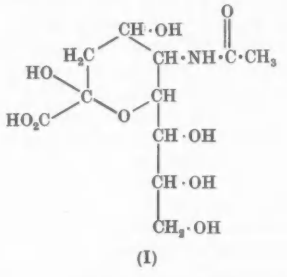
Note on nomenclature

The original and classical name, brain ganglioside, proposed by Klenk (1942) is adhered to in the present study. The name 'strandin' (Folch *et al.* 1951), given to similar material isolated from brain, is not used for the following reasons: (1) The report that strandin (Folch *et al.* 1951) does not contain appreciable neuraminic acid is now known to be in error (Chatagnon & Chatagnon, 1954; Daun, 1952; Rosenberg, Howe & Chargaff, 1956; Bogoch, 1956). (2) Other similarities in the composition of these substances have been demonstrated (Chatagnon & Chatagnon, 1954; Daun, 1952; Rosenberg *et al.* 1956; Bogoch, 1956). (3) The previously supposed difference in yield was found to be non-existent; when all the subfractions which were discarded by Klenk in his original procedure were taken into account, the yield of both substances was approx. 2 mg./g. of whole ox cerebral hemispheres (Bogoch, 1956).

RESULTS

Stepwise hydrolysis of brain ganglioside

Autohydrolysis. For recent suggestions on nomenclature for the sialic and neuraminic acids, see Blix, Gottschalk & Klenk (1957). The structure (I) proposed by Cornforth, Daines & Gottschalk (1957) for *N*-acetylneuraminic acid is probably very close to the true structure, although the molecular formula $C_{12}H_{21}O_{10}N$ for *N*-acetylneuraminic acid (Klenk & Faillard, 1954) is here used in all calculations.



(I)

It has been found that aqueous solutions of mucins and gangliosides undergo autohydrolysis, giving free neuraminic acid when heated at 100° (Blix, 1936; Folch *et al.* 1956; Bogoch, 1956). Whereas intact brain ganglioside has no reducing power, the neuraminic acid fraction liberated by autohydrolysis possesses a reducing power of 57% (expressed as glucose), and the non-diffusible residue of the ganglioside possesses no reducing power (Table 1). This suggests that the neuraminic acid is linked to the rest of the molecule through its reducing group. If samples of the autohydrolysate are taken at frequent intervals, and the reducing power is determined, the liberation of free reducing groups is seen to coincide quantitatively with the release of neuraminic acid (Bogoch, 1956). Thus it is this linkage which is cleaved during autohydrolysis.

The appearance of reducing groups is associated with the evolution of an absorption peak at 270 mμ during autohydrolysis. That this absorption may be related to a cyclization of neuraminic acid is suggested by the following experiment. An aqueous solution of crystalline neuraminic acid, obtained by treatment of diffusate I with cold methanol (1·1 mg./ml., birefringent needles, insoluble in cold methanol; N 4·5%, reducing sugar 61·4%), possesses no absorption peak at 270 mμ. If this solution is heated at 100° for 30 min. (as is done in the autohydrolysis of ganglioside), an absorption peak at this wavelength gradually appears (Fig. 1). The crystalline neuraminic acid is simultaneously converted into a pink amorphous material, now soluble in cold methanol, with unchanged reducing power. A similar change occurs spontaneously over a period of several weeks if the crystalline material is exposed to light and air at room temperature. This product, which has the same R_F as crystalline neuraminic acid, can be distinguished by the fact that it gives an immediate

Table 1. *Stepwise hydrolysis of brain ganglioside*

Procedure	Fraction	Dry wt. (as % ash-free ganglioside)	Composition of fraction (%)				
			Neuraminic acid	Nitrogen	Hexose	Reducing sugar	Galactosamine
	Ash-free ganglioside	100·0	30·3	2·9	24·0	0	10·0
Autohydrolysis at 100° for 18 min. followed by dialysis	→Diffusate I	20·8	88·0	4·42	0·74	57·0	2·5
	Residue *A*	81·0	13·0	2·7	—	—	—
Hydrolysis with 0·09 N-HCl at 100° for 50 min. followed by dialysis	→Diffusate II	17·0	33·0	4·24	21·3	57·3	14·2
	Residue *B*	64·0	3·0	1·99	31·3	—	9·6
Hydrolysis with 6 N-HCl at 100° for 5 min. followed by dialysis	→Diffusate III	23·4	—	2·36	72·6	39·3	16·6
	Residue *D*	31·8	0	—	—	—	—
Centrifuging	→Supernatant	0·14	—	—	—	—	—
	Precipitate	—	0	—	—	—	—
Solvent partition	→Upper phase (D_1)	4·3	—	2·9	17·0	2·0	0·13
	→Interphase	1·0	—	—	—	—	—
	→Lower phase	25·1	—	1·87	5·2	—	—

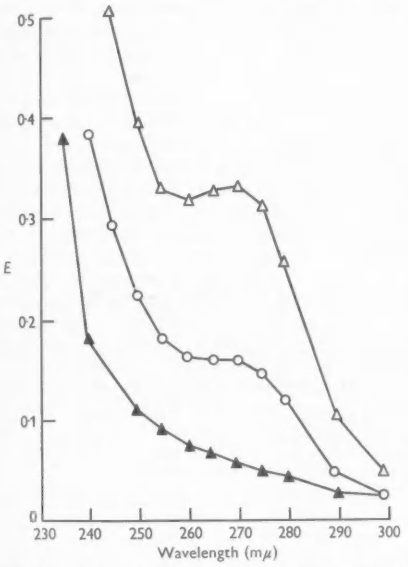


Fig. 1. The change in the ultraviolet spectrum of crystalline neuraminic acid on heating at 100°. ▲, No heating; ○, heated for 10 min.; △, heated for 30 min.

maximum red colour on spraying with Ehrlich's reagent and heating with steam, whereas crystalline neuraminic acid gives a maximum reaction only after heating for 10 min. This suggests that the change which occurs (possibly cyclization or polymerization) does not involve the reducing group.

Table 1 shows that diffusate I contains, in addition to neuraminic acid, 2·5 % of bound galactosamine (which is released in free form only after hydrolysis with N-HCl at 100° for 16 hr.) and hexose 0·74 %. This suggests that the neuraminic acid of brain ganglioside is bound to a neighbouring galactosamine molecule. Since the galactosamine of brain ganglioside is acetylated (Klenk & Lauenstein, 1953), and the amino group is thus not available for binding, it would appear likely that there is a glycosidic linkage between the reducing group of neuraminic acid and a hydroxyl group of galactosamine. Gottschalk (1956) has assumed that with some mucoproteins, neuraminic acid is linked to galactosamine by means of an *O*- or an *N*-glycosidic linkage. With brain ganglioside it would appear from the above that neuraminic acid is attached to galactosamine, and that it is attached by an *O*-glycosidic rather than an *N*-glycosidic linkage.

During the course of autohydrolysis the viscosity of the solution first decreases (to 42 % of its original value in 12 min.), increases to its original value in the next 15 min., then suddenly becomes so viscous that no flow occurs through an upright pipette with an internal diameter of 3 mm. The significance of this fact is taken up in the Discussion. The non-diffusible material remaining after autohydrolysis and dialysis is residue *A*. It accounted for 81 % of the original ganglioside (Table 1).

Hydrolysis of residue A. (1) Autohydrolysis of residue *A* (5 % aqueous solution, 100°, 30 min.) resulted in the conversion into dialysable form of only another 3 % of the original ganglioside. Thus about 30 % of the neuraminic acid of the intact molecule remains resistant to this treatment, and

requires hydrolysis with added acid to effect its removal. (2) Residue *A* was hydrolysed with added acid (0·09N-HCl, 100°, 50 min.) and then dialysed exhaustively. In this case the diffusate (diffusate II) contained a further 18·5% of the neuraminic acid, 24·2% of the galactosamine and 15% of the hexose of the intact ganglioside (Table 1). The hexose has been shown to be exclusively galactose by paper chromatography. The non-diffusible residue (residue *B*) still accounted for 64% of the original ganglioside. The analysis of residue *B* is also shown in Table 1. It may be noted that in both this and in the next hydrolytic step, there is an unavoidable destruction of neuraminic acid due to its acid lability.

Hydrolysis of residue B. Residue *B* was hydrolysed (6N-HCl, 100°, 5 min.) and then dialysed exhaustively, yielding diffusate III, which contained the balance of the recoverable neuraminic acid, galactosamine and galactose, plus some of the glucose of the original ganglioside (Table 1). It is noteworthy that this was the first point in the stepwise hydrolysis at which glucose was cloven from the ganglioside. The non-diffusible residue (residue *D*) contained the sphingosine and fatty acid of the original molecule, plus 8% of its hexose, but no appreciable galactosamine or neuraminic acid. Whereas the loss of galactosamine [in terms of the modified Elson & Morgan (1934) reaction] was 9·4% of the original up to the stage of residue *B*, a further 21·9% was lost in the brief hydrolysis of residue *B* with 6N-HCl.

Analysis of diffusates II and III. Samples of diffusates II and III were subjected to paper chromatography. In addition to distinct spots corresponding to neuraminic acid, hexosamine, glucose and galactose, two well-defined spots of slower mobility were present which corresponded to none of the above. That these slow-moving substances were oligosaccharides is suggested by the following experiments:

(1) Corresponding undeveloped areas of neighbouring duplicate strips of the same chromatogram were eluted with 5 ml. of water, and the eluates were examined by the reducing sugar and orcinol–sulphuric acid methods (Table 2). Control strips to detect substances eluted from the paper itself were taken from areas where there were no spots. The ratio 'orcinol sugar':'reducing sugar' indicated whether the sugars were free or bound. Furthermore, by determining the proportion of total hexose eluted from the area corresponding to glucose or galactose, an estimate could be obtained of the percentage of the total hexose of the fraction which was free. Diffusate II contained some 25% of its total hexose as free monosaccharide, the balance being bound in four different oligosaccharides, whereas diffusate III contained only 4% as free monosaccharide, the balance being bound in four different oligosaccharides. The recovery from the eluted spots in terms of the orcinol reaction of the original diffusate applied to the paper was 107% for diffusate II and 91% for diffusate III. From Table 2 it may be seen that spot 1 of diffusate III is an oligosaccharide. Other oligosaccharides are present in the first four spots of both diffusates, but are less apparent because of the presence of neuraminic acid and galactosamine. Thus spots 1 and 2 of diffusate II, and 2 and 3 of diffusate III, have low values for orcinol:reducing sugar because they contain neuraminic acid, which has a high reducing value but gives no hexose

Table 2. *Chromatographic demonstration of oligosaccharides and monosaccharides in diffusates II and III*

Where two spots are listed opposite neuraminic acid, these spots travelled close together opposite the single neuraminic acid spot.

Spot (in order of increasing mobility)	Orcinol sugar as galactose (μg.) (*a*)	Reducing sugar as galactose (μg.) (*b*)	Ratio *a*/*b*
Diffusate II			
(1) Neuraminic acid	280	301	0·93
(2) Neuraminic acid	40	100	0·40
(3) Tail of 2	27	83	0·33
(4) Hexosamine	88	198	0·45
(5) Tail of 4	10	30	—
(6) Galactose	150	119	1·26
Total hexose (μg.	595		
Diffusate III			
(1) Unknown	1460	440	3·32
(2) Neuraminic acid	92	80	1·15
(3) Neuraminic acid	58	75	0·77
(4) Hexosamine	84	104	0·81
(5) Galactose	15	30	—
(6) Glucose	54	30	—
Total hexose (μg.)	1763		

reaction with orcinol. Similarly, spot 4 of both diffusates has a low orcinol:reducing sugar value because of the failure of hexosamine to give an orcinol reaction. The hexose present in all of these spots is bound, for if free, it would be opposite spots 5 or 6.

(2) The presence of oligossacharides is supported by the further hydrolysis of the diffusates and chromatography of the products. Hydrolysis of diffusate II with N-HCl resulted in the disappearance of the oligosaccharide spots, and the appearance of galactose as the sole hexose. With diffusate III, both galactose and glucose were present, but they required hydrolysis with stronger acid (2N-HCl) in order to liberate the monosaccharides. Evidence for this statement is as follows: after diffusate III was hydrolysed with N-HCl (sealed tube, 100°, 16 hr.), the product could be partitioned by ion-exchange chromatography into neutral and amino-sugar fractions. The neutral sugar fraction still showed oligosaccharide spots on paper chromatography. However, when this neutral sugar fraction was treated for varying periods with 2N-HCl (sealed tube, 100°) the oligosaccharides were hydrolysed. The resultant evolution of reducing groups is shown in Fig. 2.

Amino sugar fraction of diffusates II and III. Hydrolysis of diffusate III (N-HCl, sealed tube, 100°, 16 hr.), followed by Dowex-50 chromatography, yielded an amino sugar fraction consisting of large birefringent crystals arranged spherically. These crystals represented 20·5% of diffusate III and contained 5·6% of N and 78% of hexosamine. On chromatography there was only one spot, corresponding to galactosamine.

Hydrolysis of this amino sugar fraction with 6N-HCl (100°, sealed tube, 10 min.), followed by paper chromatography, revealed that the galactosamine spot, though still present, was diminished in intensity and a new spot with 50% faster mobility, which also reacted with ninhydrin, was now present.

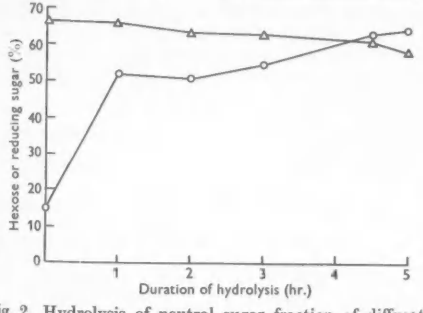


Fig. 2. Hydrolysis of neutral sugar fraction of diffusate III with 2N-HCl at 100° for varying periods: △, % hexose (as galactose); ○, % reducing sugar.

The same treatment of a pure sample of galactosamine yielded identical chromatographic results. The appearance of this second spot on treatment with 6N-HCl would appear to be related to the simultaneous loss of the ability to give the modified Elson & Morgan (1934) reaction for hexosamine which had previously been observed in the hydrolysis of residue *B* with 6N-HCl. The derivative which is formed may be a cyclized or isomerized product of galactosamine.

The hexosamine of this preparation of brain ganglioside was identified as galactosamine (see Methods), and the content of galactosamine found to be 10% by weight of the intact ganglioside.

Analysis of residue D. Residue *D* comprised the non-diffusible material remaining after hydrolysis of residue *B* with 6N-HCl (Table 1). It was a hazy, opalescent solution containing insoluble particles. The insoluble material was separated by centrifuging, then partitioned between chloroform–methanol (2:1, v/v) and water. The analyses of the resultant phases are shown in Table 1.

The upper phase (D_1) contained 2·9% N, of which 2·3% was free amino N. The hexose content was 17%. There was essentially no hexosamine, and the reducing sugar was only 2%. This suggested that D_1 contained sphingosine with some hexose attached. Evidence that this was the case follows from the results of further hydrolysis (N-HCl, sealed tube, 100°, 16 hr.). (1) The hydrolysate was centrifuged; the resultant supernatant contained 69% of the original hexose, and the precipitate contained 100% of the original N but only 10% of the original hexose (precipitate N 4·4%). (2) The reducing sugar of D_1 (as equivalents of galactose) rose from 2% before, to 19·2% after, hydrolysis. (3) Whereas chromatography of unhydrolysed D_1 revealed only one spot with very rapid mobility, and no free hexose, hydrolysed D_1 showed only glucose (no galactose).

An unexpected finding was the presence in the precipitate of hydrolysed D_1 of a reducing sugar value of 11·1% (as galactose), which was not accounted for by the hexose (orcinol) content of only 1·7%. This finding, together with the presence of 2% of reducing sugar in unhydrolysed D_1 (from which all free hexose or hexose fragments had been removed by dialysis), suggests the possibility that the sphingosine of brain ganglioside (Klenk, 1942; Rosenberg & Chargaff, 1956) is modified to the extent that it contains a reducing group. A possible function of such a reducing group is discussed in the formulation of the structure of the repeating unit.

Quantitative nature of stepwise hydrolysis

The stepwise procedure outlined was found to be reproducible. Recovery in terms of hexose and N (adding all of the subfractions) was 96·6 and

96·0 % respectively. Furthermore, the constituents were repeatedly released in the following order: neuraminic acid, galactosamine, galactose, glucose, sphingosine and fatty acids. If the hydrolytic procedure was varied, the order of release was maintained. Thus if instead of subjecting residue *B* to hydrolysis with 6 N-HCl, it was hydrolysed with 0·09 N-HCl (100°, sealed tube, 3 hr.), then dialysed exhaustively, another 11·7 % of the original ganglioside was released in diffusible form. This diffusate was found to contain 38 % of hexose (galactose only by paper chromatography) and 24·8 % of galactosamine, representing essentially the balance of the galactosamine of the intact molecule. The non-diffusible residue (B_1) still contained 27·8 % of hexose, in addition to all of the sphingosine and fatty acid. It is important to note that residue B_1 was still soluble in water, indicating that neither fatty acid nor ceramide nor cerebroside was freed in appreciable amounts, since all of these are insoluble in water.

Cleavage of a cerebroside from brain ganglioside

The hydrolyses of residues *A* and *B* with 0·09 N-HCl, which removed galactose but not glucose, suggested that a glucocerebroside might be isolated. After several attempts, optimum conditions were defined as follows: residue *A* was hydrolysed with 0·09 N-HCl (sealed tube, 100°, 16 hr.), then dialysed. The resulting non-diffusible residue (residue *C*), still water-soluble, was further hydrolysed (0·09 N-HCl, sealed tube, 100°, 5 hr.). The hydrolysate, which now contained a tan, insoluble material, was dialysed. This water-insoluble material was separated by centrifuging, dried *in vacuo* and dissolved in warm methanol–chloroform (9:1, v/v) to give a clear solution (12 % remains as insoluble black residue which is discarded). When this solution (40 mg./ml.) is allowed to cool to room temperature, birefringent spherocrystals are formed, amounting to 26–30 % by weight of the intact brain ganglioside. These crystals contain no neuraminic acid or galactosamine. The analysis, compared with that expected for a glucostearocerebroside monohydrate (Rosenheim, 1914), is as follows: [Found: C, 67·13; H, 10·95; N, 1·86; hexose (as glucose), 21·8. Calc. for $C_{42}H_{81}O_8N, H_2O$: C, 67·65; H, 11·14; N, 1·88; hexose (as glucose), 22·0 %].

The material is insoluble in water, slightly soluble in pure chloroform, very soluble in chloroform–methanol (2:1, or 3:1, v/v). It shows slight liquefaction at 165°, then melts sharply at 172–174°. The iodine number is 23·2. In chloroform–methanol (2:1, v/v) (1 mg./ml.) it is laevorotatory ($[\alpha]_D^{20} - 2{\cdot}08$). The infrared spectrum is shown in Fig. 3. A sample of the crystalline material gave the following spacings by X-ray diffraction: 2·41, 4·10, 9·1, 10·4, 15·6, 21·1, 30·6, 49·2, 55·5 and 63·5 Å.

Hydrolysis in N-HCl (sealed tube, 100°, 16 hr.) liberated the hexose quantitatively. This was shown to be exclusively glucose by paper chromatography. The remaining water-insoluble residue contained 2·3 % of N, as expected for sphingosine and fatty acid in equimolar ratio. It is yet to be established whether the base is sphingosine or dihydrosphingosine (Rosenberg & Chargaff, 1956; Carter & Norris, 1942).

The melting point, specific rotation, iodine number and elementary analysis distinguish this substance from the previously described cerebrosides phrenosine, kerasine and nervone (Deuel, 1951). A glucostearocerebroside has not previously been described. Its presence as the basic constituent of brain ganglioside would be consistent with the demonstration by Klenk (1942) that the constituent fatty acid of brain ganglioside is stearic acid. The name gangliocerebroside is proposed for this new substance.

Cleavage of a hexodicerebroside from brain ganglioside

With the demonstration that brain ganglioside could be degraded to a glucocerebroside, it became of interest to establish the nature of the linkage of

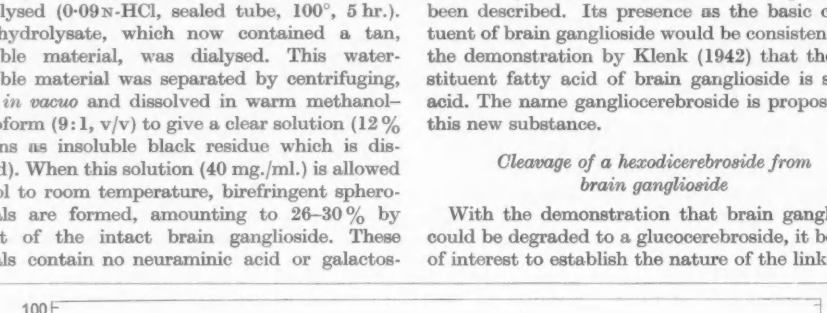


Fig. 3. Infrared spectrogram of gangliocerebroside (Perkin–Elmer double-beam automatic-recording spectrophotometer, NaCl prism).

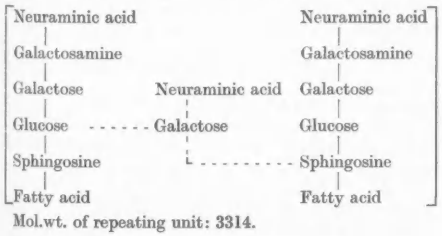
the individual cerebroside molecules to each other. Since in the preparation of the glucocerebroside no insoluble material appeared until the hydrolysis of residue *C*, it seemed likely that a fraction would be partitioned from residue *C* which was simply a dicerebroside or a dicerebroside with a hexose, or a galactosamine or possibly a neuraminic acid molecule as the bridge between the two individual cerebrosides. The analysis of such a fraction might indicate clearly which of the above four possibilities was correct. It was, of course, possible that the analyses for the above constituents would indicate a random arrangement.

When residue *C* was dried *in vacuo* and repeatedly washed with warm methanol–chloroform (9:1, v/v) a soluble fraction was obtained which crystallized in a manner similar to the ganglioceroside. The yield of this new material represented 90% by weight of residue *C* and 41% by weight of the intact brain ganglioside. Galactosamine was absent, and the neuraminic acid content was only 1·05%. The hexose content and elementary analysis strongly suggested that this substance was in fact a single or polymerized hexodicerebroside [Found: C, 66·02; H, 10·54; N, 1·77; hexose (as glucose), 30·2. Calc. for $C_{90}H_{170}O_{20}N_2,H_2O$: C, 66·01; H, 10·51; N, 1·71; hexose (as glucose), 29·8%].

This substance differed also in melting point from the glucocerebroside; it liquefied slightly at 175° and melted sharply at 182–184°.

DISCUSSION

The determination of structure by the quantitative stepwise procedure outlined suggests that what is being studied is an organized arrangement of constituents bound together in firm covalent linkage. This is indicated by the stoicheiometric appearance of reducing groups as constituents are successively cloven from glycosidic binding, and by the constant order of release of diffusible constituents. The constant order of release of constituents observed in repeated stepwise hydrolyses, i.e. neuraminic acid, galactosamine, galactose, glucose, sphingosine and fatty acids, is suggestive of a structural sequence. Furthermore, the data from the analysis of the fractions shown in parentheses present evidence for the existance of the following groupings: neuraminic acid–galactosamine (diffusate I); galactosamine–galactose–glucose (diffusates II and III; residue B_1); glucose–sphingosine (D_1); glucose–sphingosine–fatty acid; sphingosine–fatty acid and hexodicerebroside. These data, considered with the percentage composition of the constituents of the intact ganglioside, suggest the repeating unit (II) [Found: N, 2·9; neuraminic acid, 30·3; hexose, 24; galactosamine, 10. Calc. for the structure (II): N, 2·96; neuraminic acid, 30·5; hexose, 24·4; galactosamine, 10·8%].



Mol.wt. of repeating unit: 3314.

(II)

In (II) the linkages which have not yet been positively identified are shown by interrupted lines. Since there would be a shortage of reducing groups required to unite all of the members of the repeating unit in the manner suggested by the hydrolytic studies, the evidence suggesting a sphingosine molecule modified in that it possesses a reducing group is pertinent. The empirical observation that about 60% of the total neuraminic acid can be removed by autohydrolysis, but that to free the remainder requires more vigorous acidic hydrolysis, is in agreement with the more peripheral localization of 2 of the 3 moles of neuraminic acid/unit.

A repeating unit of this molecular size would appear to be unique, and presents interesting problems with regard to the spatial organization of the macromolecular polymer. By simply arranging the repeating units linearly, a fibrous structure would be realized. The physical properties suggest, however, a globular molecule. Thus in aqueous solution flow birefringence is not marked and, on autohydrolysis, the initial fall in viscosity followed by a later increase suggests the uncoiling of a globular unit, followed by the aggregation of linear fragments. A further empirical point requiring some attention is the fact that whereas brain ganglioside is remarkably soluble in water (despite its high lipid content) it is also soluble in organic solvents. The sequence indicated by this study would permit the coiling of the polymerized units into, for example, a helix, in such a way that lipid-soluble constituents could be enfolded by water-soluble constituents in aqueous solution. In organic solvents, the coiling might occur in the opposite direction, such that the lipid-soluble constituents 'faced' the solvent. It may also be noted that the extended linear form of the brain ganglioside could function admirably in transport phenomena at the nerve-cell surface, where it would be useful to present water-soluble constituents in one direction and lipid-soluble constituents in the other. The relation of neuraminic acid to the

surface receptor substance of cells for the attachment of viruses (Klenk & Lauenstein, 1952; Gottschalk, 1954, 1955; Klenk, Faillard & Lempfrid, 1955) and the demonstration that this preparation of brain ganglioside is an inhibitor of the haemagglutination reaction of influenza virus (Bogoch, 1956, 1957) are of interest in relation to the present suggestions for the overall structure.

SUMMARY

1. A preparation of brain ganglioside, homogeneous by electrophoretic and ultracentrifugal studies, has been examined by a quantitative stepwise hydrolytic procedure.

2. Diffusible products have been quantitatively separated from those which are non-diffusible at each hydrolytic step by exhaustive dialysis.

3. The diffusible and non-diffusible fractions thus obtained have been analysed in terms of dry weight, nitrogen, neuraminic acid, hexosamine, hexose and reducing sugar, partitioned by paper-chromatographic and ion-exchange procedures, and the subfractions further hydrolysed and analysed.

4. The mode of linkage of the neuraminic acid component has been demonstrated.

5. Oligosaccharide fragments were isolated, the hexoses shown to be exclusively galactose and glucose, and the hexosamine, galactosamine.

6. The galactosamine content has been found to be 10%, and a reaction of galactosamine on heating with concentrated acid has been observed.

7. A glucocerebroside has been cloven from the intact ganglioside, characterized and named gangliocerebroside.

8. A hexodicerebroside has also been isolated.

9. The constant order of release of constituents, together with the evidence regarding the structure of isolated fragments, has been used to formulate the structure of the repeating unit of the molecule.

10. Observations have been made relevant to the overall structure and orientation of the macromolecular glycolipid.

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Pathways of Thyroid Phosphorus Metabolism

THE PHOSPHOLIPIDS OF SHEEP THYROID

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There is much evidence for the importance of phosphorus in the metabolism and function of the thyroid gland. Kocher (1902) first described an inverse relationship between the contents of iodine and phosphorus in this tissue. More recently, Dempsey & Singer (1946) have presented histochemical evidence for marked fluctuations in the activities of acid and alkaline phosphatases and of hexose phosphatases in the gland during periods of varying thyroid activity, and Borell & Holmgren (1949) have demonstrated that the uptake of radioactive phosphorus by the thyroid *in vivo* is increased by pituitary thyrotropin. With preparations *in vitro*, Morton & Schwartz (1953) have reported that pituitary thyrotropin selectively increases the incorporation of ^{32}P into the phospholipids of ox-thyroid slices, and Freinkel & Ingbar (1955*a*) have shown that the first step in the synthesis of thyroid hormone, namely the accumulation of I^- ion, is dependent upon energy derived from oxidative phosphorylation. Despite these data, detailed inquiry into the composition and metabolic pathways of thyroid tissue has not hitherto been made.

The present paper deals with phosphoglyceride metabolism. The phospholipids and related compounds which occur in sheep thyroid have been characterized by recently developed chromatographic techniques (Dawson, 1954, 1955*a*). Moreover, these methods have been used to study the incorporation *in vitro* of both [^{32}P]phosphate and [^{14}C]glycerol into the individual phosphoglycerides and their metabolic intermediates in sheep-thyroid slices. The studies have yielded information about the metabolic pathways of the phospholipids, and the active turnover of these components suggests that the phospholipids may occupy a central role in the function of this gland.

EXPERIMENTAL

Materials

[^{32}P]Orthophosphate and [^{14}C]glycerol were obtained from the Radiochemical Centre, Amersham, Bucks. The glycerol is prepared substantially by the method of Karnovsky & Gidez (1952) and thus consists of DL-[1-^{14}C]-glycerol (Karnovsky, Hauser & Elwyn, 1956). On paper chromatography, in the solvents described below, the glycerol ran as a single radioactive spot.

Thyroid tissue was obtained from sheep which were 12 months of age or older.

Methods

Fractionation experiments. Intact thyroid glands were excised from sheep within 5 min. of killing and plunged directly into liquid O_2. For each analysis a weighed portion (0·7–1 g.) of frozen thyroid was added to 4 ml. of water, contained in a graduated centrifuge tube in a boiling-water bath. After 2 min., the boiled tissue and water were homogenized in a Potter–Elvehjem ground-glass homogenizer and returned to the original centrifuge tube. The homogenizer was washed with 7 ml. of ethanol and the washings were combined with the aqueous homogenate and dispersed with a glass rod. After centrifuging, the turbid supernatant solution was shaken for 5 min. with 40 ml. of $CHCl_3$; the mixture then separated into water- and $CHCl_3$-soluble phases.

The water-soluble phase was passed through a column of Amberlite IRC 50 (H^+ form) resin to remove cations which would otherwise interfere with the subsequent paper chromatography. The column was washed twice with 10 ml. portions of water and the total effluent was cleared of turbidity and concentrated by shaking with 3 vol. of *iso*butanol. The aqueous phase was neutralized to pH 7 with aq. NH_3 soln., concentrated *in vacuo* below 50° and quantitatively applied to washed filter paper for two-dimensional chromatography in phenol–aq. NH_3 soln. (descending) and ethanol–aq. NH_3 soln. (ascending) (Dawson, 1955*a*, *b*).

The $CHCl_3$-soluble phase was combined with the insoluble residue which had been suspended in 10 ml. of ethanol and transferred quantitatively from the centrifuge tube to an Erlenmeyer flask. The suspension was boiled for 20 min., cooled to 0° and filtered through glass wool. The filtrate was shaken with 2 vol. of 0·1 N-HCl in order to remove any acid-soluble, phosphorus-containing contaminants (Dawson, 1955*a*). The purified lipid extract was taken to dryness *in vacuo* and the phospholipids were examined by two-dimensional chromatography after mild alkaline hydrolysis (Dawson, 1954). This mild hydrolysis of the phospholipids selectively removes the fatty acid residues from the phosphoglycerides and leaves the individual water-soluble glycerylphosphoryl-base units intact.

Incubation experiments. Thyroid glands were excised from sheep within 5 min. of killing, and wrapped in cellophan bags, placed on cracked ice and transported to the

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laboratory in Dewar flasks. Thyroid slices, 0·3–0·4 mm. thick, were prepared by the method of Deutsch (1936), and placed in Petri dishes containing ice-cold 0·15 M-NaCl.

For incubation, two types of suspending media were employed: (*a*) phosphate medium, composed of 0·131 M-NaCl, 5 mM-KCl, 1·2 mM-$MgSO_4$, 0·8 mM-$CaCl_2$ and 10 mM-sodium phosphate buffer (pH 7·4), and (*b*) phosphate-free medium composed of the same constituents except for the substitution of 10 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·4) for the sodium phosphate buffer.

Thyroid slices were quickly blotted and introduced in 320–350 mg. amounts into 2·7 ml. of phosphate-free medium contained within the main compartment of chilled Warburg vessels. Side arms of the Warburg vessels held 0·3 ml. of the phosphate medium, containing 25–35 μc of inorganic [^{32}P] phosphate or 15 μc of [1-^{14}C]glycerol (1 μmole of glycerol/μc).

Details of the incubation and manometric techniques have been described previously (Freinkel & Ingbar, 1955*b*). All incubations were started within 90 min. of killing the animal. The slices were shaken at 38° with pure O_2 in the gas phase. After gassing and temperature equilibration under these conditions for 10 min., the phosphate medium in the side arm was tipped into the phosphate-free medium contained within the main compartment. This facilitated the introduction of phosphorus of known specific radioactivity into the system at a desired time. At intervals during the subsequent 4 hr., slices were removed from the flasks, quickly blotted, weighed, frozen in liquid O_2 and stored to await fractionation as described previously. Suspending media were centrifuged immediately at 4° and the supernatant solutions were stored at −18°.

Chromatography. Sheets of Whatman no. 1 filter paper were well washed with 2 N-acetic acid before chromatography. The following solvent systems were employed: phenol (A.R.) saturated with 0·1% aq. NH_3 soln.; ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (61:29:10, by vol.) (Dawson, 1955*b*); trichloroacetic acid (10%, w/v) in 2-methylpropan-2-ol–water (62:38, v/v) (the trichloroacetic acid was removed from the dried chromatograms by washing with diethyl ether; Dawson, 1954); *n*-butanol–2-ethoxyethanol–ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (40:40:15:12·5:40, by vol.) (Meredith & Sammons, 1952). Descending chromatography was employed for the phenol–aq. NH_3 soln., and ascending for all the other solvents.

After chromatography, the phosphorus-containing compounds were localized by spraying with the acid-molybdate reagent of Hanes & Isherwood (1949) and by irradiating the dried sheets with ultraviolet light (Bandurski & Axelrod, 1951). Preliminary spraying with 0·1% ninhydrin in acetone did not interfere with the molybdate reaction. This facilitated the identification of ninhydrin-reacting phospholipid intermediates as well as providing reference amino acid spots which helped in the localization of other intermediates. In addition, all radioactive compounds were localized by routine radioautography of chromatograms in contact with Kodirex X-ray Film.

Phosphorus estimation and radioactive assay. A modification of the method of Berenblum & Chain (1938) was employed for estimating small quantities of phosphorus, and larger amounts were measured by the method of Fiske & Subbarow (1925). The phosphorus-containing spots on each chromatogram were cut out together with a paper blank of equivalent weight from the area immediately surrounding each spot. In the fractionation experiments and in the studies with ^{32}P, the paper spots and blanks were introduced into 0·9 ml. of 72% perchloric acid A.R. and oxidized to orthophosphate by heating them with suitable shielding on an electric Kjeldahl-digestion rack. The digests were assayed for phosphorus and the ^{32}P contained in them was estimated directly in an M-6 liquid counter tube (20th Century Electronics). Specific radioactivities are expressed as counts of ^{32}P min./μg. of P, and were corrected to an initial specific radioactivity of 100 000 counts/min. of ^{32}P/μg. of P in the phosphate medium contained within the side arms.

In experiments with [1-^{14}C]glycerol, the phosphorus-containing spots and paper blanks were eluted by direct extraction with 5 ml. of 2% aq. NH_3 soln. Eluates were dried *in vacuo* at room temperature over concentrated H_2SO_4 and redissolved in 2·5 ml. of water. Samples (1 ml.) were placed on aluminium planchets and dried for radioactive assay at infinite thinness with a mica end-window Geiger–Müller tube. Equal volumes were digested with perchloric acid and analysed for phosphorus. Specific radioactivity values were expressed as counts of ^{14}C/μg. of P/min.

In all radioactive analyses, sufficient counts were observed to reduce the probable error of the measurement to less than 3%.

RESULTS

Examination of sheep-thyroid tissue for phospholipids and intermediates of their metabolism

In eight fresh sheep-thyroid glands, the water-soluble phosphorus averaged 201·5 ± 54·1 μg. of P/g. wet wt. and constituted 18·2 ± 5·4% of the total glandular phosphorus. Inorganic phosphorus comprised 19·4 ± 5·9% of the water-soluble phosphorus and averaged 39·4 ± 14·5 μg. of P/g. wet wt. All values are mean ± S.D.

The following water-soluble phosphoglyceride intermediates were identified in two-dimensional chromatograms prepared from aqueous extracts of fresh sheep-thyroid tissue: α-glycerophosphoric acid (GPA), phosphorylethanolamine (PE), phosphorylcholine (PC), glycerylphosphorylethanolamine (GPE) and glycerylphosphorylcholine (GPC) (Fig. 1). Identification was based on (*a*) identical migrations of synthetic markers added directly to the aqueous extracts, and (*b*) parallel migration of synthetic markers concurrently chromatographed along the outer margins of the filter paper. PE and GPE were further identified on the basis of their reaction with ninhydrin. (The markers were supplied by Dr R. M. C. Dawson.)

The phenol–aq. NH_3 soln. and ethanol–aq. NH_3 soln. solvents effectively separated GPE, GPC and PC from the other water-soluble phosphorus-containing components of sheep-thyroid tissue. Recovery of added GPE, GPC or PC introduced into the initial homogenate was greater than 80%. Therefore it is possible to use chromatographic

techniques to derive minimum estimates of the concentrations of these compounds (Table 1).

It was observed that, 24–48 hr. after spraying with acid molybdate, two brownish grey spots developed in previously unmarked areas. R_F values for these compounds were 0·13 and 0·37 in phenol–aq. NH_3 soln. and 0·71 and 0·83 in ethanol–aq. NH_3 soln. respectively. GPC reference markers which were chromatographed simultaneously in the same solvent systems had R_F values of 0·87 and 0·84 respectively. The larger of the two spots, which remained closest to the origin in both phenol–aq. NH_3 soln. and ethanol–aq. NH_3 soln., has been provisionally identified as free inositol since inositol markers displayed identical chromatomatographic mobilities and colour reactions.

The phosphorus in the lipid extracts prepared from eight fresh sheep-thyroid glands averaged $191{\cdot}5 \pm 30{\cdot}1$ μg. of P/g. wet wt. and constituted $17{\cdot}3 \pm 5{\cdot}8$ % of the total glandular phosphorus. After mild alkaline hydrolysis, $77{\cdot}6 \pm 7{\cdot}3$ % of the lipid phosphorus was recovered in the aqueous phase. Resolution of the water-soluble hydrolysis products by two-dimensional chromatography disclosed spots which corresponded to the mobilities which Dawson (1954) has described for the glycerylphosphoryl residues in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and phosphoinositides. The respective spots were identified as GPC, GPE, glycerylphosphorylserine, GPA, and a compound containing glycerol, phosphorus and inositol residues. In three experiments, the chromatographic separations of the hydrolysis products of the phosphatidylserine and the phosphoinositide were incomplete. Glycerophosphoric acid could be demonstrated in only four of eight experiments. Whether this compound represents a fractionation artifact or whether thyroid phosphatidic acid is present in too limited amount for the routine detection of its hydrolysis products cannot be assessed from the available data.

Since the mild alkaline hydrolysis of thyroid phospholipids left only about 20 % of the lipid phosphorus intact, some of which would be sphingomyelin and other alkali-stable phospholipids, the distribution of thyroid phosphoglycerides can be estimated by chromatographic partition of the water-soluble phosphorus. In this way, 52, 25, 11, 9 and 3 % of the total phosphorus in the five identified spots could be attributed to the respective hydrolysis products of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphoinositide and phosphatidic acid.

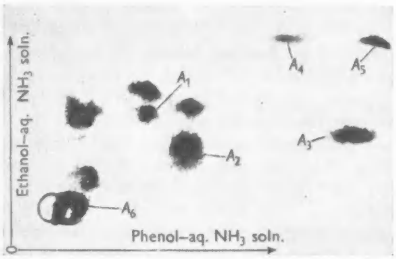

Fig. 1. Two-dimensional chromatogram of the water-soluble phosphorus-containing compounds in sheep-thyroid gland. Spots were localized by spraying with acid molybdate and by irradiating the dried sheets with ultraviolet light. A_1, Glycerophosphoric acid; A_2, phosphorylethanolamine; A_3, phosphorylcholine; A_4, glycerylphosphorylethanolamine; A_5, glycerylphosphorylcholine; A_6, inorganic phosphorus.

Table 1. *Concentration of phospholipid intermediates in aqueous extracts of sheep-thyroid tissue*

PC, phosphorylcholine; GPC, glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine. Values are % of total, water-soluble organic phosphorus. Total water-soluble organic P was determined on aqueous extracts of 0·7–1·0 g. (wet wt.) of fresh sheep-thyroid tissue. Individual compounds determined by chromatographic resolutions of aqueous extracts.

Expt. no.	Total water-soluble organic P (mg./g. wet wt.)	PC (%)	GPC (%)	GPE (%)
1	165	3·4	4·2	1·5
2	87	5·0	3·6	0·8
3	134	3·2	2·2	1·3
4	169	3·0	3·6	1·2

Incorporation of ^{32}P into the phosphoglycerides and their intermediates in sheep-thyroid slices

Surviving sheep-thyroid slices incorporated ^{32}P into all of the phosphorus-containing compounds which had been identified in the earlier fractionation studies. Labelled GPA, PC and PE were disclosed by brief radioautographic exposure of chromatograms of the water-soluble extracts; more prolonged radioautography was required for detection of the sparsely labelled GPC and GPE. Spots corresponding to the water-soluble hydrolysis products of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and the phosphoinositide were seen in radioautographs of chromatograms of the hydrolysed labelled lipid extracts (Fig. 2). In addition, two unidentified radioactive spots were seen in these radioautographs (cf. L_4 and L_5, Fig. 2), but these are not peculiar to the thyroid (N. Freinkel & R. M. C. Dawson, unpublished work).

Specific radioactivities of the individual phosphoglycerides differed considerably. Incorporation of ^{32}P was greatest into phosphoinositide. At all times during incubation, the phosphoinositide contained two to ten times more $^{32}P/\mu g.$ of P than any other lipid component. Representative values from one of nine experiments with ^{32}P are presented in Table 2. During serial observations, the specific radioactivities of the individual phosphoglycerides were found to increase at different rates (Table 2). Within the limits of the analytical techniques, systematic changes in the concentration of individual phosphoglycerides or certain phosphoglyceride intermediates could not be detected during incubation *in vitro* despite a 10–20% increase in the concentration of inorganic phosphorus in the suspending medium. On the basis of such evidence for equilibrium conditions, isotopic techniques were employed to assess the interrelationships between the individual phospholipids and water-soluble intermediates of lipid metabolism. Slices from the same sheep-thyroid gland were incubated in a number of flasks and, at regular intervals, tissues were removed and fractionated for the concurrent estimation of the specific radioactivities of water- and chloroform-soluble phosphorus compounds. Results from one of three experiments are given in Figs. 3 and 4. At all times throughout incubation, the specific radioactivities of the water-soluble phosphorylated bases, PE and PC, exceeded those of phosphatidylethanolamine and phosphatidylcholine, whereas the specific radioactivities of the water-soluble phosphodiesters, GPE and GPC, were less than those of the phosphoryl units in the corresponding phosphoglycerides.

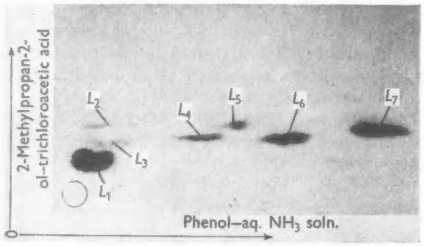

Fig. 2. Radioautograph of a two-dimensional chromatogram of an alkaline hydrolysate of the lipids extracted from 340 mg. (wet wt.) of sheep-thyroid tissue. Before fractionation, thyroid slices were incubated for 4 hr. in 3 ml. of suspending medium containing 25 μc of $NaH_2{}^{32}PO_4$. L_1, Hydrolysis product of the thyroid phosphoinositide; L_2, glycerophosphoric acid; L_3, glycerylphosphorylserine; L_4, unidentified; L_5, unidentified; L_6, GPE; L_7, GPC.

Incorporation of [1-^{14}C]glycerol into the phosphoglycerides of thyroid slices

After the incubation of sheep-thyroid slices with DL-[1-^{14}C]glycerol, ^{14}C could be demonstrated in the glycerylphosphoryl residues of all of the phosphoglycerides. Specific radioactivities of the individual phosphoglycerides after 1–4 hr. incubation differed but were not as variable as the values which had been observed with ^{32}P (Table 2). Experiments were performed in which slices from the same sheep-thyroid gland were incubated in different Warburg flasks with either ^{32}P or [1-^{14}C]-glycerol. Data from one of three such paired-isotope experiments are presented in Table 2. The ratios of the $^{14}C/^{32}P$ specific radioactivities of the individual phospholipids showed considerable variation at any single instant. During serial analyses, ^{14}C specific radioactivities did not increase as rapidly as the ^{32}P values.

Table 2. *Incorporation of* DL-[1-^{14}C]*glycerol and* $NaH_2{}^{32}PO_4$ *into the glycerophosphoryl units of the phospholipids of sheep-thyroid slices*

Slices (330–350 mg. initial wet wt.) from the same sheep-thyroid gland were incubated in 3 ml. of suspending medium containing either 30 μc of $NaH_2{}^{32}PO_4$ or 15 μc of DL-[1-^{14}C]glycerol. Slices were removed after 2 and 4 hr. Average values for q_{O_2} (μl. of O_2/mg. initial wet wt./hr.) during the 4 hours were 0·40, 0·35, 0·35 and 0·33 respectively. Specific radioactivities are expressed as counts of ^{32}P or $^{14}C/\mu g.$ of P/min. Values for ^{32}P have been corrected to an initial specific radioactivity of 100 000 counts/$\mu g.$ of P/min. in the side arms of the Warburg vessels.

	Specific radioactivities							
	^{32}P		^{14}C		$^{14}C/^{32}P$		$\frac{4\text{ hr.}}{2\text{ hr.}} \times 100$	
Phospholipid	2 hr.	4 hr.	2 hr.	4 hr.	2 hr.	4 hr.	^{32}P	^{14}C
Phosphatidylcholine	426	1504	83	123	0·195	0·082	353	148
Phosphatidylethanolamine	218	666	31	54	0·142	0·081	305	174
Phosphatidylserine	175	428	16	29	0·091	0·068	244	181
Phosphatidic acid	973	1605	—	14	—	0·015	165	—
Phosphoinositide	2344	7262	64	105	0·027	0·014	310	164

Verification of an inositol-containing phospholipid in the sheep thyroid

The highly radioactive spot which had been identified as a phosphoinositide on the basis of R_F characteristics was localized by radioautography and eluted with water. The eluate was hydrolysed with 5N-HCl for 36 hr. at 100°. The acid was removed by evaporation *in vacuo* and the hydrolysis products were resuspended in water and resolved by the chromatographic technique of Meredith & Sammons (1952). Free inositol, glycerophosphoric acid and inorganic phosphate were demonstrated, thus verifying the presence within sheep-thyroid gland of a phospholipid containing glycerol, inositol and phosphorus residues.

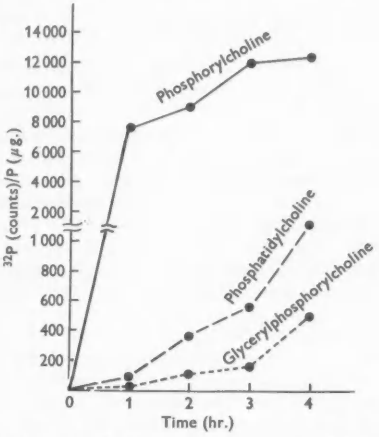

Fig. 3. The metabolism of lecithin (phosphatidylcholine) in surviving sheep-thyroid tissues. Slices weighing 300–330 mg. were incubated in 3 ml. of suspending medium containing 27 μc of $NaH_2{}^{32}PO_4$ and were removed at intervals for analysis of the compounds in aqueous and lipid extracts. Average values for q_{O_2} during the 4 hr. incubation were 0·33, 0·30, 0·27 and 0·26 respectively.

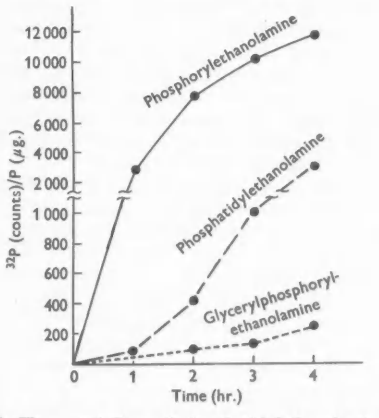

Fig. 4. The metabolism of phosphatidylethanolamine in sheep-thyroid tissue. Slices were incubated with 27 μc of $NaH_2{}^{32}PO_4$ and removed at intervals for analysis of the compounds in aqueous and lipid extracts.

DISCUSSION

Although the synthesis of thyroid hormone has been extensively studied, few data are available on the metabolism and the composition of thyroid tissue. In the present studies, attention has been focused upon the phospholipids of the sheep-thyroid gland. Five separate phosphoglycerides have been demonstrated in lipid extracts. Whether one of these (i.e. phosphatidic acid) occurs naturally in limited quantities or constitutes a fractionation artifact has not been definitely answered. In addition, it has been shown that selected phosphoglyceride intermediates account for a large proportion of the organic, water-soluble phosphorus of sheep-thyroid tissue. Extrapolation of all of the findings to other species must await further investigation.

During incubation *in vitro*, sheep-thyroid slices can incorporate ^{32}P into all of the phosphorus-containing compounds. For the phospholipids, such complete labelling is in marked contrast with the limited incorporations which have been obtained with certain other tissue preparations (Dawson, 1954). All of the thyroid phosphoglycerides exhibited different ^{32}P specific radioactivities throughout incubation, and serial fractionations disclosed that these specific radioactivities changed at different rates. This heterogenous labelling of the phosphoglycerides of sheep-thyroid tissue is similar to Dawson's (1955*b*) findings for rat liver *in vivo* and supports the growing impression that valid estimates of the turnover of lipid phosphorus cannot be derived on the basis of unfractionated phospholipid extracts. It was found, however, that the phosphoinositide was consistently more heavily labelled with ^{32}P than any other phosphoglyceride, which contrasts markedly with the findings for liver.

The incorporation of ^{32}P has facilitated an examination of the possible pathways of phosphorus metabolism of lecithin and phosphatidylethanolamine. Serial fractionation of incubated sheep-thyroid slices revealed that the phosphorus in phosphatidylethanolamine and phosphatidylcholine was never as heavily labelled as the corresponding water-soluble phosphorylated bases, PE and PC. At the same time, the specific

radioactivities of the phosphoglycerides were consistently greater than those of the water-soluble phosphodiesters, GPE and GPC. The relatively unlimited availability of radioactive label in the suspending medium precludes rigorous application of the precursor–product relationships of Zilversmit, Entenmann & Fishler (1943) to systems *in vitro*. Nevertheless, the specific radioactivities of the phosphorylated bases could not have been so great if PE and PC had become labelled as a consequence of breakdown of phosphoglyceride. Similarly, the sparsely labelled phosphodiesters, GPE and GPC, could not have served as the principal source of phosphoglyceride phosphorus. Therefore, if PE, PC, GPE and GPC constitute water-soluble intermediates in the metabolism of phosphatidylethanolamine and phosphatidylcholine, a precursor role must be ascribed to the phosphorylated bases, whereas the phosphodiesters must be derived chiefly from phosphoglyceride breakdown. Dawson (1955*a*, 1956*a*) has reached similar conclusions about phospholipid pathways in rat liver *in vivo*. The recent demonstration of phospholipase *B* in the sheep-thyroid gland (Dawson, 1956*b*) strengthens the above evidence for the phosphodiester route of phosphoglyceride catabolism.

The evidence for the precursor function of the phosphorylated bases is reinforced by the experiments with DL-[1-^{14}C]glycerol. In these studies ^{14}C could be demonstrated in all of the thyroid phosphoglycerides. That some of the ^{14}C may have represented labelled choline, ethanolamine or serine rather than unaltered glycerol cannot be excluded; but, on the basis of preliminary experiments, a significant occurrence of this seems unlikely. The observed differences in the ^{14}C/^{32}P specific radioactivity ratios of the individual phosphoglycerides would argue against a common phosphorylated glycerol precursor (such as glycerophosphoric or phosphatidic acid) for the phosphorus in thyroid phosphoglycerides. Rather, the findings would support the role of the phosphorylated bases as the principal donors of phosphoglyceride phosphorus, presumably by activation through cytidine triphosphate (Kennedy & Weiss, 1956).

Despite all of the foregoing evidence for an active phospholipid metabolism within the sheep-thyroid gland, the physiological ends which this metabolism serves remain to be elucidated. It is, however, becoming increasingly apparent that the phospholipids are concerned with the structural elements of multi-enzyme systems in tissues, such as oxidative phosphorylation (Cooper & Lehninger, 1956), and consequently an active phospholipid metabolism may be needed to maintain this organization. Moreover, it is possible that the phospholipids may play an important role in the active transport of ions across membranes (Solomon, Lionetti & Curran, 1956). Both of these phenomena are now known to be highly important in maintaining the thyroid gland in full functional activity (Schachner, Franklin & Chaikoff, 1943; Freinkel & Ingbar, 1955*a*, *b*).

SUMMARY

1. Sheep-thyroid gland has been examined by chromatographic techniques for the presence of phosphoglycerides and water-soluble intermediates of their metabolism. In aqueous extracts, the presence of glycerophosphoric acid, phosphorylcholine, phosphorylethanolamine, glycerylphosphorylcholine, glycerylphosphorylethanolamine and inositol has been demonstrated. In lipid extracts, the presence of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and a phosphoinositide has been shown.

2. During incubation of sheep-thyroid slices, [^{32}P]orthophosphate was incorporated into all of the phosphorus-containing compounds. Specific radioactivities of the individual phospholipids varied greatly; the phosphoinositide became more heavily labelled than any other phosphoglyceride. Concurrent fractionation of aqueous and lipid extracts disclosed that the ^{32}P specific radioactivities of the phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine were greater than those of the water-soluble phosphodiesters, glycerylphosphorylcholine and glycerylphosphorylethanolamine, and less than the specific radioactivities of the water-soluble phosphorylated bases, phosphorylcholine and phosphorylethanolamine.

3. The incorporation of DL-[1-^{14}C]glycerol *in vitro* into all of the phosphoglycerides of sheep-thyroid tissue has been demonstrated, but the specific radioactivities were not as heterogeneous as the corresponding ^{32}P values.

4. From these isotopic results, certain conclusions have been reached about the probable pathways of phosphoglyceride metabolism in sheep-thyroid tissue.

It is a pleasure for the author to acknowledge his indebtedness to Sir Rudolph A. Peters, F.R.S., and Dr R. M. C. Dawson of the A.R.C. Institute of Animal Physiology, Babraham, Cambridgeshire, for their constant encouragement, stimulating advice and generous help in the preparation of this manuscript. The author would also like to express his appreciation for the technical assistance of Miss Colleen Tebbut and for the fellowship support of the National Foundation for Infantile Paralysis, U.S.A.

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Synthesis of α-Methyl- and β-Methyl-DL-Cystine

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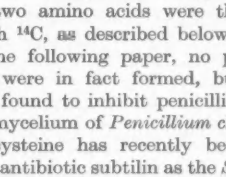
In view of the utilization of L-cystine as a precursor of the β-lactam ring of penicillin (Arnstein & Grant, 1954) it was of interest to study penicillin biosynthesis in the presence of compounds related to cystine. For this purpose, α-methyl- and β-methyl-cystine were required and the present paper deals with their synthesis. For some experiments, it was necessary to use isotopic labelling in order to detect any possible conversion of the substituted cystines into $C_{(5)}$- or $C_{(6)}$-substituted penicillins, which might be biologically inactive, and these two amino acids were therefore also labelled with ^{14}C, as described below. As will be shown in the following paper, no penicillin-like compounds were in fact formed, but α-methylcystine was found to inhibit penicillin production by washed mycelium of *Penicillium chrysogenum*.

β-Methylcysteine has recently been found to occur in the antibiotic subtilin as the *S*-(L-2-amino-2-carboxyethyl) derivative (I) (Alderton, 1953), but the configuration of the asymmetric β-carbon atom was not elucidated. The stereochemistry of β-methylcysteine is thus of some interest in connexion with the structure of this antibiotic.

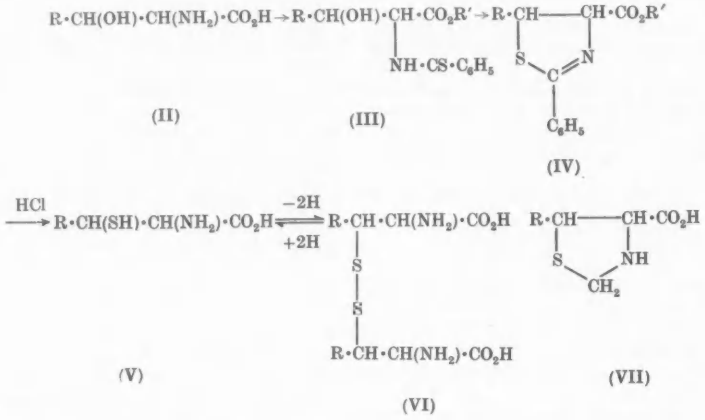


(I)

Synthesis of β-methyl-DL-cysteine and its disulphide from allo*threonine and threonine*

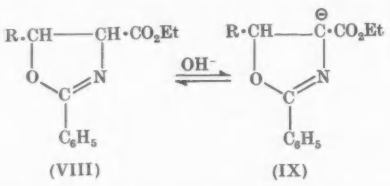
Of the four possible stereoisomers of β-methylcysteine (V, R = CH_3) which correspond in configuration to the L- and D-enantiomorphs of threonine and *allo*threonine, both pairs of DL-isomers (named isomers *A* and *B* of thiothreonine) have been synthesized by Carter, Stevens & Ney (1941), but their stereochemical relationship was not investigated. It was hoped to clarify this problem in the present work by applying the recent synthesis of cystine (VI, R = H) from serine (II, R = H) via the thiobenzoyl ester (III, R = H, R′ = C_2H_5) and thiazoline ester (IV, R = H, R′ = C_2H_5) derivatives (Crawhall & Elliott, 1951) to the preparation of β-methylcystine (VI, R = CH_3) from DL-threonine and DL-*allo*threonine (II, R = CH_3).

In view of the inversion at the β-carbon atom during ring closure to the thiazoline (IV, R = CH_3, R′ = C_2H_5), it was anticipated that this synthesis would yield the *erythro* and *threo* forms of DL-β-methylcysteine (V, R = CH_3) respectively. It was



found, however, that both threonine and *allo*threonine unexpectedly gave rise to the same α-amino-β-mercaptobutyric acid (V), although, under identical conditions, a better yield was obtained from the latter. This amino acid (V) was identified as isomer *B* of thiothreonine (Carter *et al.* 1941) by mixed melting-point determinations and X-ray powder photographs of the thiazolidine (VII, $R = CH_3$) obtained by reaction with formaldehyde (Cook & Heilbron, 1949).

It seems likely that in the synthesis from threonine the intermediate *cis*-2-phenylthiazoline ethyl ester (IV, $R = CH_3$; $R' = C_2H_5$) was converted into the *trans*-isomer. Such an isomerization is well known (see Elliott, 1953) for the corresponding oxazolines (VIII, where R may be methyl, phenyl,



pentadecyl or hydroxymethyl), but a strong base is required for this reaction. The ester anion (IX) is probably formed as an intermediate, whilst steric factors are believed to be responsible for the preferential formation of the *trans*-oxazoline from this intermediate (Elliott, 1949), and a similar mechanism may account for the conversion of *allo*threonine and threonine into the same thiolamino acid.

The overall yield (20 %) of β-methylcystine from thiobenzoyl*allo*threonine ethyl ester is considerably lower than that obtained in the analogous synthesis of cystine (47 %; Crawhall & Elliott, 1951), probably due to formation of relatively large amounts of the oxazoline. Although the oxazoline was not isolated, its presence is indicated by the recovery of substantial quantities of α-amino-β-hydroxybutyric acid from the acid hydrolysis of the cyclization product. Moreover, cyclization of thiobenzoylhydroxyaspartic acid ester has been shown to result in the formation of the oxazoline as well as the thiazoline (Hauptmann & Berl, 1955). Substitution of the carbon atom carrying the hydroxyl group thus seems to favour cyclization to the oxazoline rather than to the thiazoline, at least when thionyl chloride is used. This is reminiscent of the reaction of amino alcohols with carbon disulphide and alcoholic alkali, where substitution of the carbinol carbon atom also results in oxazoline formation, as discussed by Crawhall & Elliott (1952).

The assumption that synthesis of α-amino-β-mercapto acids from the corresponding hydroxyamino acids via a thiazoline results only in inversion of configuration at the β-position, which has been made, for example, in a recent paper on the preparation of cystine-ββ′-dicarboxylic acid from *erythro*hydroxyaspartic acid (Hauptmann & Berl, 1955), is thus not valid. It is suggested that, in the present work, the *trans*-thiazoline was formed from both *allo*threonine and threonine; in the latter case, inversion of the β-configuration must have been followed by one at the α-position, probably by the mechanism discussed above. The formation of only one form of β-methylcystine would thus be accounted for and it is concluded from comparison of this compound with the *A* and *B* isomers of thiothreonine (Carter *et al.* 1941) that these are the *erythro*- and *threo*-isomers of β-methylcysteine

respectively. In the synthesis of cystine-ββ′-dicarboxylic acid mentioned above, both the *erythro* configuration of the starting material and saponification of the thiazoline ester with strong potassium hydroxide would favour formation of the *trans*-thiazoline carboxylic acid (see Elliott, 1953) and designation of the configuration of the product as *threo* (Hauptmann & Berl, 1955) is therefore not invalidated by the present work.

Synthesis of α-methyl-DL-cystine

Benzylthioacetone (cf. Wahl, 1922) was converted into the amino nitrile, which was hydrolysed without purification of the crude product, giving *S*-benzyl-α-methyl-DL-cysteine in 70 % yield. After the present work was completed, Potts (1955) reported a synthesis of the latter, also from benzylthioacetone but via the hydantoin instead of the amino nitrile, the yield by this method being about 65 %.

Reduction of the *S*-benzyl group by sodium in liquid ammonia (Wood & du Vigneaud, 1939) and oxidation of the product by air in the presence of a trace of ferric chloride at pH 8 gave α-methyl-DL-cystine. By using $K^{14}CN$ for synthesis of the amino nitrile, α-methyl-DL-[*carboxy*-^{14}C]cystine was prepared.

One noteworthy property of α-methylcysteine, and probably also of other α-substituted amino acids, is the very slow colour reaction with ninhydrin. Although the α-hydrogen atom of an amino acid is apparently not essential for reaction with ninhydrin (Spenser, Crawhall & Smyth, 1956), substitution of the α-position by a methyl group evidently has a profound effect on the rate of reaction.

EXPERIMENTAL

Radioactivity measurements. All samples were counted on 1 cm.^{2} polythene disks at infinite thickness, by using a thin-end-window Geiger-Müller counter (Popják, 1950). The specific radioactivities were obtained by direct comparison with a poly[^{14}C]methyl methacrylate standard containing 1 μc/g., which was obtained from the Radiochemical Centre, Amersham.

Paper chromatography. All R_F values refer to descending chromatograms on Whatman no. 1 paper. The solvent systems had the following composition (by vol.): *A*, phenol–water, 5:2 (with NH_3); *B*, pentan-1-ol–pyridine–water, 7:7:6; *C*, butanol–acetic acid–water, 63:10:27.

Synthesis of DL-[carboxy-^{14}C]allo*threonine*

[carboxy-^{14}C]*Butyric acid.* Propyl iodide (34 g., 0·2 mole, of material dried over $CaCl_2$ and freshly redistilled, b.p. 102–104°) was dissolved in ether (500 ml.) and added slowly to ether-washed Mg turnings (4·8 g.). When the initial reaction had subsided, the mixture was warmed on a water bath for 30 min. The yield of Grignard reagent, which was determined by adding a small portion of the above solution to excess of 0·1 N-HCl and back-titrating with 0·1 N-NaOH, was 90 %. Carbonation of the Grignard reagent was carried out essentially as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949), by using $^{14}CO_2$ from $Ba^{14}CO_3$ (12 g., 0·15 mole; 1·5 mc). When absorption of CO_2 was complete, the product was poured on crushed ice (750 g.) and acidified with conc. H_2SO_4 (18 ml.) in water (150 ml.). The aqueous phase was saturated with NaCl and extracted with ether (3 × 200 ml.). After drying with Na_2SO_4, the ether was evaporated under reduced pressure. The residue was distilled at atmospheric pressure, giving 7 g. (53 %, based on CO_2) of product, b.p. 160–162°.

iso*Butyl* α-*bromo*-[carboxy-^{14}C]*butyrate.* The labelled butyric acid was brominated in the presence of dry P (1 g.) by slow addition of dry Br_2 (8·5 ml.). The mixture was heated on the steam bath for 5 hr., kept overnight at room temp., and added dropwise to *iso*butanol (22·3 ml.) with stirring and cooling. The solution was boiled under reflux for 15 min., cooled and added to saturated $NaHCO_3$ (50 ml.) in a separating funnel. The bromo ester was separated and the aqueous solution extracted twice with ether, the ether extracts being mixed with the ester. The solution was dried ($CaCl_2$), the ether was evaporated and the residue distilled, giving 13·7 g. (78 %) of *iso*butyl α-bromo-[*carboxy*-^{14}C]butyrate, b.p. 128–132° at 50 mm. Hg.

iso*Butyl* [carboxy-^{14}C]*crotonate.* The above bromo ester was added dropwise to boiling diethylaniline (20 g.) (Hunter & Popják, 1951), the last traces being added with a further 2 ml. of diethylaniline. The mixture was boiled under reflux for 23 hr., cooled, acidified with 6 N-HCl (9 ml.) and extracted twice with ether. The ether solution was dried (K_2CO_3 + $CaCl_2$) and the residue, after removal of ether, was distilled at 150 mm. Hg, giving 3·2 g. (37 %) of product, b.p. 116–124°.

[carboxy-^{14}C]*Crotonic acid.* The above ester (3·2 g.) was refluxed with N-NaOH (35 ml.) for 2·5 hr. After cooling, the mixture was extracted twice with a little $CHCl_3$, acidified with 6 N-HCl (6 ml.) and extracted with ether. The ether solution was dried (Na_2SO_4) and the ether was evaporated in a stream of air. The residue was dried *in vacuo* over P_2O_5. The yield of crude [*carboxy*-^{14}C]crotonic acid, m.p. 76–78°, was 1·5 g. (78 %), but on recrystallization from light petroleum (b.p. 60–80°; 5 ml.) only 0·88 g. was recovered. After successive additions of non-radioactive crotonic acid (0·8 g.; 1·0 g.; 2·0 g.), further crops of less active material (0·85 g., 1·3 g., and 1·7 g. respectively) were obtained, corresponding to a total recovery of 89 % on crystallization.

α-*Bromo*-β-*hydroxy*[carboxy-^{14}C]*butyric acid.* The labelled crotonic acid (4·6 g.) obtained above was dissolved in 160 ml. of water and gaseous Br_2, generated from Br_2 (9 g.) by a stream of N_2, was passed through the solution for 5·5 hr. (cf. Carter & Zirkle, 1949). The solution was concentrated *in vacuo* (bath temp. 40–50°) to 20 ml. and extracted with benzene (5 × 40 ml.) to remove αβ-dibromobutyric acid, m.p. 88° (Found: Br, 65·3, Calc. for $C_4H_6O_2Br_2$: Br, 65·0 %). After evaporation of the aqueous layer, the residue failed to crystallize and was therefore dissolved in water (30 ml.) and again extracted with benzene (5 × 30 ml.). Evaporation of the aqueous solution to dryness (bath temp. <50°) yielded crude α-bromo-β-hydroxybutyric acid, m.p. 70–75°. After recrystallization from benzene by addition of light petroleum (b.p. 60–80°), α-bromo-β-hydroxy[*carboxy*-^{14}C]butyric acid (3·1 g., 32 %; m.p. 84–86°; mixed m.p. with αβ-dibromobutyric acid, 63–66°) was obtained.

DL-[carboxy-^{14}C]allo*Threonine*. The above α-bromo-β-hydroxy[*carboxy*-^{14}C]butyric acid (3 g.) was mixed with carrier (5 g.) and aminated at room temp. with a mixture of $(NH_4)_2CO_3$ (24 g.), conc. aq. NH_3 soln. (80 ml.) and water (32 ml.). After 24 hr. the mixture was heated at 60–65° for 6 hr. and then evaporated to dryness. The residue was dissolved in hot water (25 ml.) and boiling ethanol (250 ml.) was added. After standing overnight at 4°, 4·8 g. of product was obtained. Recrystallization from hot water (15 ml.) by adding boiling ethanol (150 ml.) gave 4·5 g. (87%) of DL-[*carboxy*-^{14}C]*allo*threonine, m.p. 241–242°, specific radioactivity 11·2 μc/g. The overall chemical and radiochemical yields from $Ba^{14}CO_3$ were 3·0 and 3·4% respectively.

Conversion of allo*threonine into β-methylcysteine (thiothreonine, isomer* B*) and its disulphide*

DL-[*carboxy*-^{14}C]*allo*Threonine (2·1 g., 1·34 μc/m-mole, 23·3 μc) was suspended in dry ethanol (50 ml.). Dry HCl gas was passed through for 15 min., when the ethanol was boiling. After cooling in ice, the solution was saturated with HCl and left at room temp. overnight. The ethanol was removed *in vacuo*, the residue was dried in a desiccator and seeded. The crystalline product was washed with dry ether; yield 3·2 g. (99%). The ester hydrochloride was dissolved in pyridine (50 ml.) and triethylamine (3·6 g.) and (thiobenzoylthio)acetic acid (4·2 g., prepared as described by Crawhall & Elliott, 1951) were added. After 24 hr., the reaction mixture was added to water (500 ml.) and extracted with ether (150 ml., 75 ml. and 75 ml.). The ethereal extracts were combined, washed with water, 2N-HCl (7 × 6 ml.), saturated $NaHCO_3$ and water (10 ml.). The ether layer was separated, dried (Na_2SO_4) and the ether was evaporated. The residue was dried in a desiccator overnight and crystallized from ether–light petroleum (b.p. 40–60°). The yield of *N*-thiobenzoyl*allo*threonine ethyl ester, m.p. 80–82°, was 3·1 g. (66%). The solid was finely powdered and added to redistilled $SOCl_2$ (5·8 ml.). After 15 min., the $SOCl_2$ was evaporated *in vacuo* at <30° and the residue was triturated with ether, when it crystallized. The crystalline solid was washed with ether, dissolved in $CHCl_3$ and treated with charcoal. After filtering, the $CHCl_3$ was evaporated, giving a cryst. product (3·5 g.), which was hydrolysed by boiling under reflux with 3N-HCl (120 ml.) for 18 hr. Benzoic acid was removed by extraction with ether and the aqueous solution was concentrated to dryness by evaporation *in vacuo*. Paper chromatography (solvent *B*) showed the presence of approximately equal quantities of β-methylcysteine and *allo*threonine. The crude product was therefore chromatographed on a column (approx. 2·5 cm. diam.) containing 250 g. of Zeo-Karb 225 resin (Permutit Co. Ltd., London) in the H^+ form, 1·5N-HCl being used as eluent. Fractions of about 10 ml. each were collected; fractions no. 50–70 contained *allo*threonine together with small amounts of an unidentified ninhydrin-positive impurity (detected by paper chromatography with solvent system *B*), whilst β-methylcysteine was eluted later (fractions no. 110–200). Evaporation of the appropriate fractions yielded *allo*threonine hydrochloride (0·52 g.) and β-methylcysteine hydrochloride (0·64 g., 33%) respectively. The radioactivity of this material, measured after combustion to CO_2 and conversion into $BaCO_3$, was found to be 8·1 μc/g. (1·38 μc/m-mole), which is in good agreement with that of the starting material. The overall chemical and radiochemical yields from *allo*threonine were 19·8 and 22·3% respectively.

A portion of the amino acid hydrochloride was converted into the free amino acid by adsorption on Zeo-Karb 215 resin in the H^+ form (about 10 mg. of resin/mg. of amino acid) and elution with aq. 0·2N-NH_3 soln. This procedure also resulted in oxidation of the sulphydryl group to the disulphide. The eluate was evaporated to dryness *in vacuo* and the residue crystallized from water by adding ethanol, giving *β-methylcystine*, m.p. 193–194° (Found: C, 35·3; H, 6·6; N, 9·7; S, 24·9. $C_8H_{16}O_4N_2S_2$ requires C, 35·8; H, 6·0; N, 10·45; S, 23·9%). The R_F values were 0·58 in solvent *A*, 0·03 in solvent *B* and 0·06 in solvent *C*.

In another experiment, non-radioactive β-methylcystine (1·5 g.) was dissolved in conc. HCl (5 ml.) and reduced with Sn (1 g.). After dilution with approx. 50 ml. of water, the Sn was precipitated with H_2S, and the solution filtered and evaporated to dryness. The amino acid hydrochloride was converted into the free amino acid by treatment with Zeo-Karb 215 resin as described previously. Owing to the rapid oxidation of sulphydryl groups by air at an alkaline pH, this procedure resulted in the re-oxidation of β-methylcysteine to β-methylcystine (Found: C, 35·8; H, 6·2; N, 9·7; S, 24·7%). Several further attempts to obtain more satisfactory analyses for nitrogen and sulphur were unsuccessful, although no impurities could be detected by paper chromatography (solvent systems *A*, *B* and *C*).

Conversion of β-methylcysteine into 4-carboxy-5-methylthiazolidine (cf. Cook & Heilbron, 1949). Non-radioactive β-methylcysteine hydrochloride (0·59 g.), which had been prepared from non-radioactive *allo*threonine and chromatographed as described above, but with Dowex 50 ion-exchange resin (Microchemical Specialities Co., Berkeley, California), was dissolved in water (1·1 ml.), formalin (0·5 ml.) was added and the solution was kept at room temp. for about 18 hr. Pyridine (0·5 ml.) and ethanol (1 ml.) were added and the solution was cooled to 4°, when a crystalline product, m.p. 204°, separated. After recrystallization from water by addition of ethanol, pure 4-carboxy-5-methylthiazolidine (0·1 g., m.p. 209–210°) was obtained (Found: C, 40·9; H, 6·5; N, 8·9; S, 21·95. Calc. for $C_5H_9O_2NS$: C, 40·8; H, 6·1; N, 9·5; S, 21·8%). A simultaneous mixed m.p. determination with 4-carboxy-5-methylthiazolidine prepared from the *B* isomer of thiothreonine (cf. Carter *et al.* 1941) showed no depression, and the mixed m.p. with the corresponding derivative of thiothreonine, isomer *A*, was 192°.

Conversion of threonine into β-methylcysteine

DL-Threonine (10 g.) was suspended in dry ethanol (200 ml.) and dry HCl was passed in until the solution boiled. After cooling to 0°, the solution was saturated with HCl and kept at room temp. overnight. The ethanol was evaporated *in vacuo* and the residue was freed from excess of HCl in a vacuum desiccator over NaOH; the yield of crude DL-threonine ethyl ester hydrochloride was 16·1 g. The ester was dissolved in pyridine (200 ml.), redistilled triethylamine (17 g.) and (thiobenzoylthio)acetic acid (20 g.) were added and the mixture was allowed to stand at room temp. overnight. The reaction mixture was poured into water (2 l.) and extracted with ether (500 ml., 250 ml., 250 ml.) and the ether extracts were washed with water

(3 × 25 ml.), 2N-HCl until the aqueous phase was acid, saturated $NaHCO_3$ (25 ml.) and water (25 ml.). The ether solution was then dried (Na_2SO_4) and evaporated. The residue was crystallized from ether by adding light petroleum (b.p. 40–60°), giving *N-thiobenzoyl-DL-threonine ethyl ester*, m.p. 83–85° (12·8 g., 58%). After two further recrystallizations the m.p. was 84–85° (Found: C, 58·2, H, 6·6; N, 5·2; $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2%).

The above ester (8·3 g.) was dissolved in $CHCl_3$ (200 ml.), the solution was cooled in ice and PCl_5 (15 g.) was added in small portions with shaking (see Hauptmann & Berl, 1955). After 30 min. at 0° and 30 min. at room temp., the $CHCl_3$ solution was extracted with sat. $NaHCO_3$, dried (Na_2SO_4) and evaporated. The crude thiazoline was hydrolysed and the product chromatographed on Zeo-Karb 225 resin, essentially as described above for the separation of β-methylcysteine from *allo*threonine. β-Methylcysteine hydrochloride was obtained by evaporation of the appropriate fractions from the column; the yield was 1·07 g. (20%).

Cyclization of thiobenzoylthreonine ethyl ester (8·2 g.) with $SOCl_2$ under the same conditions as described for thiobenzoyl*allo*threonine ethyl ester, followed by hydrolysis and chromatography on Zeo-Karb 225 resin as above, also gave β-methylcysteine hydrochloride, but in lower yield (0·42 g., 8%).

Reaction with formaldehyde. A portion of the above product (0·52 g.) in water (0·97 ml.) was mixed with formalin (0·44 ml.) and kept overnight at room temp. Pyridine (0·44 ml.) and ethanol (0·88 ml.) were added and the solution was cooled, when 4-carboxy-5-methylthiazolidine (0·11 g.) crystallized. After recrystallization from water by addition of ethanol, 80 mg. of pure product, m.p. 206°, was obtained (Found: C, 40·4; H, 6·1; N, 9·4. Calc. for $C_5H_9O_2NS$: C, 40·8; H, 6·1; N, 9·5%). There was no depression of m.p. when this material was mixed with the thiazolidine from β-methylcysteine prepared from *allo*threonine or with the thiazolidine from thiothreonine, isomer *B*, but a mixture with the thiazolidine from thiothreonine, isomer *A*, had m.p. 194°.

Preparation of DL-[carboxy-^{14}C]*thiothreonine, isomer A*

[carboxy-^{14}C]*Hippuric acid.* Redistilled benzoyl chloride (6·3 ml., 0·066 mole) and 1·92N-NaOH (28·6 ml., 0·055 mole) were added over 20 min. with stirring to [*carboxy*-^{14}C]glycine (4·1 g., approx. 200 μc; 0·055 mole) in N-NaOH (55 ml.), the rate of addition of the solution of NaOH being about four times that of the benzoyl chloride. After a further 30 min. the mixture was acidified to Congo red, cooled and the product was filtered off, washed with water and dried; yield, 9 g. (92%); specific radioactivity, 20·6 μc/g.

4-Ethylidene-2-phenyl-[5-^{14}C]*oxazolone.* The radioactive hippuric acid (9 g., 0·05 mole), acetic anhydride (16 ml., 0·15 mole) and basic lead acetate (7·6 g.) (Finar & Libman, 1949) were added to redistilled acetaldehyde (4·4 g., 0·1 mole), the mixture was shaken at 23° for 18 hr., poured into water (150 ml.) and extracted with benzene (2 × 50 ml.). The benzene layer was washed once with water (10 ml.) and evaporated, giving 7·2 g. (77%) of crude product, m.p. 82–85°. After recrystallization from ethanol by adding water, 5·5 g. (58%), m.p. 87–90°, was obtained (specific radioactivity, 17·4 μc/g.).

2-Benzamido-3-benzylthio-[carboxy-^{14}C]*butyric acid.* Addition of phenylmethanethiol to the above oxazolone, hydrolysis and benzoylation of the product was carried out as described by Carter *et al.* (1941). The yield of mixed *A* and *B* isomers was 6·6 g. (69%), having specific radioactivity 9·94 μc/g. The *A* isomer was separated from this mixture via the phenylethylamine salt (Carter *et al.* 1941), yield, 1·72 g. (26%), m.p. 151–152°; specific radioactivity, 10·2 μc/g.

[carboxy-^{14}C]*Thiothreonine* (A *isomer*). The foregoing product (1·72 g., 17·5 μc) was mixed with non-radioactive 2-benzamido-3-benzylthiobutyric acid (10·7 g.) and hydrolysed by boiling with formic acid (163 ml.)–conc. HCl (188 ml.)–water (213 ml.) for 3 hr. [*carboxy*-^{14}C]*S*-Benzylthiothreonine *A* was isolated essentially as described by Carter *et al.* (1941), the crude product being recrystallized by dissolving it in 2N-HCl (30 ml.) and neutralizing with 15N-NaOH (4 ml.), followed by solution in aq. 6N-NH_3 soln. (40 ml.) and evaporation of the NH_3 in a desiccator containing HCl; yield, 5 g. (11·7 μc, 67%), m.p. 198°; specific radioactivity, 2·34 μc/g. Reduction of the *S*-benzyl group with Na and isolation of thiothreonine hydrochloride (2 g.) were carried out as described by Carter *et al.* (1941). The hydrochloride was converted into the free amino acid by dissolving it in ethanol and adding pyridine, giving 0·85 g. (28%; specific radioactivity, 3·1 μc/g.) of [*carboxy*-^{14}C]thiothreonine *A*. The R_F values of the disulphide, obtained by oxidation at an alkaline pH, were identical with those of the B form in all three solvents.

Preparation of α-methyl-DL-cystine

Benzylthioacetone. Chloroacetone (20·9 g.) was added with stirring to the sodium derivative of phenylmethanethiol (25·9) in ethanol (160 ml.). After 3 hr., the ethanol was evaporated, the residue was extracted with ether and a saturated aqueous solution of $NaHSO_3$ was added to the ether layer with shaking. The bisulphite compound was filtered off, washed with ethanol and ether and dried. It was decomposed by warming with 2N-HCl and the ketone was extracted with ether. After evaporation of the ether the residue was distilled at 0·03 mm. Hg, giving benzylthioacetone (23·7 g., 58%), b.p. 108–110° (Wahl, 1922, gives b.p. as 155–156°/17 mm. Hg.). The 2:4-dinitrophenylhydrazone derivative was prepared by adding the ketone (100 mg.) to 2:4-dinitrophenylhydrazine (150 mg.), dissolved in conc. H_2SO_4 (0·3 ml.) and ethanol (3 ml.). After dilution with water (3 ml.), the precipitate (0·14 g., 70%) was filtered off, dried and recrystallized from ethanol. *Benzylthioacetone* 2:4-*dinitrophenylhydrazone* had m.p. 132° (Found: C, 53·1; H, 4·5; N, 15·8; $C_{16}H_{16}O_4N_4S$ requires C, 53·3; H, 4·5; N, 15·6%).

S-Benzyl-α-methylcysteine. Benzylthioacetone (17·8 g.) was added with stirring during 0·5 hr. to a mixture of NH_4Cl (11·5 g.), NaCN (10·4 g.), conc. aq. NH_3 soln. (70 ml.) and ethanol saturated with gaseous NH_3 (40 ml.). After shaking overnight at room temp., the amino nitrile was extracted with ether. The ether was evaporated and the oily residue hydrolysed by refluxing with conc. HCl (50 ml.) for 2·5 hr. The solution was evaporated to dryness, the residue was dissolved in water and the pH was adjusted to 6, when *S*-benzyl-α-methylcysteine crystallized; yield 15·6 g. (70%), m.p. 244° (decomp.). This material gave a blue colour with ninhydrin only after 5–10 min. at 100° in 0·1M phosphate buffer, pH 6·9 (Found: C, 58·4; H, 6·6;

N, 6·3; S, 13·8. Calc. for $C_{11}H_{15}O_3NS$: C, 58·7; H, 6·7; N, 6·2; S, 14·2%). The R_F values were 0·91 in solvent *A*, 0·54 in solvent *B* and 0·70 in solvent *C*.

S-Benzyl-N-formyl-α-methylcysteine. *S*-Benzyl-α-methylcysteine (5 g.) was formylated by the procedure of Wood & du Vigneaud (1939), giving 2·8 g. of crude product. After two recrystallizations from 30% ethanol, pure *S-benzyl-N-formyl-α-methylcysteine*, m.p. 154°, was obtained (Found: C, 57·2; H, 5·9. $C_{12}H_{15}O_3NS$ requires C, 56·9; H, 6·0%).

α-Methyl-DL-cystine. *S*-Benzyl-α-methylcysteine (5 g.) was dissolved in liquid NH_3 (200 ml.) and Na (approx. 1 g., 2 equiv.) was added slowly with stirring until a permanent blue colour appeared. The colour was just discharged with NH_4Cl and more NH_4Cl (2·4 g., 2 equiv.) was added. Ammonia was evaporated, the residue was extracted thoroughly with ether, dissolved in water and oxidized at pH 8, after addition of a trace of $FeCl_3$, by passing a stream of air overnight. The pH was adjusted to 6 with HCl and the solution was evaporated to a small volume, when *α-methyl-DL-cystine*, m.p. 260° (decomp.), (1·49 g., 50%) crystallized. After recrystallization from water–ethanol the product had m.p. 260° (decomp.) (Found: C, 35·7; H, 6·0; N, 10·4; S, 22·9. $C_8H_{16}O_4N_2S_2$ requires; C, 35·8; H, 6·0; N, 10·4; S, 23·85%). From the mother liquors of the first crystallization a second crop (0·3 g.) was obtained by precipitation as the Hg complex (Neuberg & Kerb, 1912), regeneration to the amino acid hydrochloride with H_2S, conversion into the free amino acid by addition of pyridine and recrystallization from water–ethanol (Found: C, 36·2; H, 6·1; N, 10·6; S, 23·3.) The R_F values were 0·62 in solvent *A*, 0·03 in solvent *B* and 0·06 in solvent *C*.

α-Methyl-DL-[carboxy-^{14}C]cystine. In the synthesis of the labelled amino acid from K^{14}CN, obtained from the Radiochemical Centre, Amersham, an attempt was made to avoid the use of excess of labelled cyanide by adding K^{14}CN (2·3 g., 35 μc) to a mixture of benzylthioacetone (6·5 g.), NH_4Cl (4·3 g.), conc. aq. NH_3 soln. (26 ml.) and saturated ethanolic NH_3 soln. (14·6 ml.). After 18 hr. at room temperature with shaking, excess of non-isotopic KCN (2·9 g.) was added and the reaction mixture was shaken at room temperature for a further 24 hr., when the product was isolated as described previously. The chemical yield of *S*-benzyl-α-methyl-DL-[*carboxy*-^{14}C]cysteine (3·3 g., 40·5%; m.p. 243°) was, however, somewhat less than in the trial experiment with unlabelled material, and the recovery of ^{14}C was very poor (3·0 μc, 9%). The *S*-benzyl-α-methyl-DL-[*carboxy*-^{14}C]cysteine (1·7 g., 1·5 μc) was treated with Na in liquid NH_3 and α-methyl-DL-[*carboxy*-^{14}C]cystine was isolated as before. After recrystallization from water–ethanol, 0·2 g. (22%) of the labelled amino acid (specific radioactivity, 1·4 μc/g.) was obtained. On paper chromatography this product and unlabelled α-methyl-DL-cystine had identical R_F values in solvent systems *A*, *B* and *C*.

SUMMARY

1. A synthesis of β-methyl-DL-cystine from DL-*allo*threonine is described. Cyclization of *N*-thiobenzoyl*allo*threonine ethyl ester with thionyl chloride gave 4-carboxy-5-methyl-2-phenylthiazoline, which was hydrolysed by acid to β-methyl-DL-cysteine (thiothreonine, α-amino-β-mercaptobutyric acid). This amino acid was found to be identical with isomer *B* of thiothreonine, previously synthesized by Carter *et al.* (1941).

2. By a similar series of reactions DL-threonine was also converted into isomer *B* of thiothreonine.

3. The significance of formation of the same isomer of thiothreonine from both threonine and *allo*threonine is discussed. It is suggested that isomers *A* and *B* of thiothreonine have the *erythro* and *threo* configuration respectively.

4. The synthesis of α-methyl-DL-cystine is reported.

5. The preparation of DL-[*carboxy*-^{14}C]*allo*threonine, β-methyl-DL-[*carboxy*-^{14}C]cystine and α-methyl-DL-[*carboxy*-^{14}C]cystine is also described.

I should like to thank Professor V. du Vigneaud, Department of Biochemistry, Cornell University Medical College, New York, for the samples of 4-carboxy-5-methylthiazolidine from isomers *A* and *B* of thiothreonine, which were used as reference compounds in the present work. I am also very grateful to the following members of the staff of the National Institute for Medical Research: Mrs O. Kennard for the X-ray powder photographs; Dr D. F. Elliott for helpful advice and discussion on the synthesis of β-methylcystine; Mr E. J. Toms and Mr E. Stevens for technical assistance.

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The Biosynthesis of Penicillin

7. FURTHER EXPERIMENTS ON THE UTILIZATION OF L- AND D-VALINE AND THE EFFECT OF CYSTINE AND VALINE ANALOGUES ON PENICILLIN SYNTHESIS*

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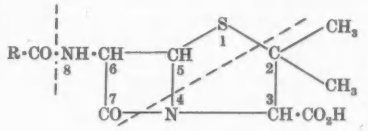
The biogenetic origin of the entire penicillin ring structure with the exception of the thiazolidine nitrogen atom is now well established. The side-chain nitrogen atom, the sulphur atom and the carbon and hydrogen atoms of the β-lactam ring of penicillin are derived from L-cystine (Arnstein & Grant, 1954*b*; Arnstein & Crawhall, 1957) whereas the carbon chain of the penicillamine moiety is synthesized from valine (Arnstein & Grant, 1954*a*; Stevens, Vohra & DeLong, 1954; Arnstein & Clubb, 1957) as shown in formula (I). It has, however, not



(I; R = $C_6H_5 \cdot CH_2 \cdot$ in benzylpenicillin)

been shown conclusively whether the immediate precursor is D- or L-valine or the keto acid (α-oxo-*iso*valeric acid) (see Arnstein & Clubb, 1957). Moreover, little is known about the structure of intermediates or the mechanism of reactions involved in penicillin biosynthesis.

In the present work, the effect of amino acids related to cystine and valine on the formation of penicillin has been studied for two reasons: first, it seemed possible, in view of the retention of the α-hydrogen atom and one of the β-hydrogen atoms of cystine during its conversion into penicillin (Arnstein & Crawhall, 1957), that α- or β-substituted cystines might give rise to penicillins substituted at $C_{(6)}$ or $C_{(5)}$ of the β-lactam ring respectively; secondly, selective inhibition of one step in penicillin biosynthesis by analogues of cystine or valine might result in accumulation of intermediates and thus facilitate a study of the biosynthetic mechanism.

The effect of amino acid analogues on penicillin biosynthesis was tested with the washed mycelium of *Penicillium chrysogenum*. Of several cystine analogues, only α-methylcystine inhibited penicillin production markedly and a similar effect was obtained with two amino acids related to valine, isoleucine and α-methylvaline. A more detailed study indicated that penicillin biosynthesis was inhibited by α-methylvaline without any apparent decrease in the synthesis of mycelial protein. Moreover, the conversion of D-valine into penicillin was decreased more than that of L-valine, indicating that D-valine is not a normal penicillin precursor.

EXPERIMENTAL

Methods

Organism and maintenance of cultures. Two strains of *Penicillium chrysogenum* were used: WIS 51/20, kindly supplied by Dr M. Lumb, Boots Pure Drug Co. Ltd., and WIS 51/20 F 3, a more stable mutant, which was obtained from Professor M. J. Johnson, University of Wisconsin. Subcultures and spore inocula were prepared as described previously (Arnstein & Grant, 1954*b*).

Details of fermentations. All fermentations were carried out in a stirred aerated jar fermenter containing 1–1·5 l. of synthetic medium (Jarvis & Johnson, 1947) under conditions essentially identical with those used earlier (Arnstein & Clubb, 1957), except that with *P. chrysogenum* WIS 51/20 F3 addition of potassium phenylacetate (0·1%/24 hr.) was begun 24 hr. instead of 40 hr. after inoculation. A vegetative inoculum was used except in the experiments described in Table 2, where the medium was inoculated with spores. The vegetative inoculum was prepared by adding 5 ml. of a suspension of washed spores from one medical flat (36 in.² area) in water (10 ml.) to 40 ml. of the synthetic medium described by Jarvis & Johnson (1947) in a 500 ml. conical flask and incubating at 24° on a rotary shaker (250 rev./min.). At the end of the incubation period (approx. 50 hr.) the entire culture was added to 1·5 l. of fermentation medium.

Washed mycelium experiments. Washed mycelium, prepared from 48–72 hr.-old fermentations as described by Halliday & Arnstein (1956), was incubated at 24° on a rotary shaker (250 rev./min.) at a concentration of 0·1 g. (wet wt.)/ml. of medium. The mycelial dry wt. was usually 10–20% of the wet wt. The composition of the incubation mixtures was as follows: medium *A*, 0·05M-glucose (0·9%), 0·15M-potassium phosphate buffer (pH 6·9), 0·005%

* Part 6: Arnstein & Crawhall (1957).

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potassium phenylacetate (pH 6·9); medium *B*, 0·1 M-potassium phosphate buffer (pH 6·9), 0·005% potassium phenylacetate (pH 6·9); medium *C*, medium *B* + 1% of glucose.

Penicillin bioassay. Penicillin was estimated by the cup-plate method with *Bacillus subtilis* (ICI strain, NCTC 8241) as test organism (Humphrey & Lightbown, 1952).

Isotope estimations. ^{14}C was estimated by counting 'infinitely thick' samples on 1 cm.^{2} polythene disks with a thin end-window Geiger–Müller tube (Popják, 1950), a 1 cm.^{2} disk of poly[^{14}C]methyl methacrylate, obtained from the Radiochemical Centre, Amersham, Bucks, being used as reference standard. The standard error of all counts is less than 5%.

For analyses of ^{15}N a 60° mass spectrometer was used as in earlier work, the accuracy being $\pm 0{\cdot}003$ atom % excess of ^{15}N (Arnstein & Grant, 1954*b*).

Isolation and degradation of penicillin. At the end of the fermentation, the mycelium was filtered off and washed with 0·1 M-potassium phosphate buffer (pH 6·9). In the washed-mycelium experiments with ^{14}C-labelled valine, non-isotopic sodium benzylpenicillin and DL-valine were added as carrier before filtration of the medium. Penicillin was extracted from the combined filtrate and washings with ether at pH 2 and isolated as the *N*-ethylpiperidine salt (cf. Arnstein & Grant, 1954*a*, *b*). The salt was purified by recrystallization from $CHCl_3$–acetone until the radioactivity remained unchanged and was degraded by acid hydrolysis as described recently by Arnstein & Clubb (1957).

Valine was isolated from mycelial protein as in previous work (Arnstein & Clubb, 1957).

Estimation of the uptake of valine by mycelium. The aqueous solution remaining after extraction of penicillin from the filtered-culture fluid was passed through a column of Zeo-Karb 215 ion-exchange resin (Permutit Co. Ltd., London) in the H^+ form, sufficient resin being used to retain all the amino acids (approx. 5 g. of resin/m-mole of valine + isoleucine or α-methylvaline). After washing with water, the resin was eluted with dil. aq. NH_3 soln. (usually 0·02 or 0·1 M) and the eluate containing the amino acids was evaporated to dryness. The residue was weighed accurately and its specific radioactivity was then determined by counting an infinitely thick sample in the usual way. The total radioactivity in the eluted amino acid fraction, which was assumed to be due to valine, was then calculated. In Expts. 1 and 5 (Table 4), valine isolated by the above method was recrystallized from aq. ethanol and the total radioactivity was obtained by multiplying its specific radioactivity by the weight of carrier valine added (40·1 mg.). In both, the amount of valine taken up by the mycelium is defined as the difference between the total radioactivity added initially as [*carboxy*-^{14}C]valine and that remaining in the valine or in the amino acid fraction isolated from the medium at the end of the incubation period.

MATERIALS

Amino acids related to cystine. The synthesis of ^{14}C-labelled and unlabelled β-methyl-DL-cystine (thiothreonine disulphide) and α-methyl-DL-cystine has been described in the preceding paper (Arnstein, 1958). The β-methyl*threo*-DL-[U-^{14}C]cystine (U indicates uniformly labelled) used in Expt. 2 (Table 2) was prepared from a mixture of L[U-^{14}C]-threonine (obtained from the Radiochemical Centre, Amersham) and unlabelled DL-threonine. Since inversion of the α-carbon atom probably occurs in this synthesis (Arnstein, 1958), the isotopic carbon would be expected to be present only in the D-enantiomorph of the product.

S-Methyl-L-cysteine was prepared by methylation of the sodium derivative of L-cysteine with methyl iodide in liquid NH_3 (du Vigneaud, Loring & Craft, 1934). *S*-Benzyl-L-cysteine was prepared by Dr D. Morris by the method of Wood & du Vigneaud (1939).

N-Methyl-L-cystine was synthesized by reducing 4-carboxythiazolidine, prepared from L-cysteine and formaldehyde (Ratner & Clarke, 1937), with Na in liquid NH_3 (Cook & Heilbron, 1949), followed by oxidation of the *N*-methyl-L-cysteine to the disulphide with air in the presence of a trace of $FeCl_3$ (Bloch & Clarke, 1938).

DL-Cysteic acid was prepared by Dr P. T. Grant (cf. Arnstein & Grant, 1954*b*).

Samples of L- and D-penicillamine were kindly supplied by Dr J. Wilkinson, Wellcome Research Laboratories. The thiol compounds were oxidized to the disulphides by aeration of solutions at pH 8 in the presence of a trace of $FeCl_3$ until no purple colour was obtained with nitroprusside.

L-Methionine was a gift from Dr T. S. Work.

Amino acids related to valine. N-Methylvaline has been synthesized from α-bromo*iso*valeric acid and methylamine (Friedmann, 1908), but in the present synthesis methylation of *p*-toluenesulphonyl-DL-valine was used, followed by hydrolysis of the *p*-toluenesulphonyl group. A solution of toluene-*p*-sulphonyl chloride (19 g.) in ether (50 ml.) was added to DL-valine (5·85 g.) in 2 N-NaOH (27·5 ml.) and the mixture was shaken at room temp., 25 ml. portions of 2 N-NaOH being added after 1 and 2 hr. After a further 1 hr., the aqueous layer was separated and acidified and the crude product was filtered off. After recrystallization from dil. ethanol, p-*toluenesulphonyl*-DL-*valine*, m.p. 168–169° (10·6 g., 79%), was obtained (Found: N, 4·9. $C_{12}H_{17}O_4NS$ requires N, 5·2%). The *p*-toluenesulphonyl-DL-valine (5·43 g.) was dissolved in 2 N-NaOH (40 ml.) and heated with methyl iodide (5·68 g.) in a sealed tube at 65–68° for 2 hr. The cooled mixture was acidified with HCl, when a yellow oil separated. The oil was dissolved in $NaHCO_3$ and reprecipitated with HCl. After some hours, 4·72 g. (83%) of crude *N*-methyl-*p*-toluenesulphonyl-DL-valine crystallized. The crude material (4·4 g.) was heated with conc. HCl (20 ml.) in a sealed tube at 105° for 24 hr. After cooling to 0°, the *p*-toluenesulphonic acid was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in ethanol and pyridine was added, when a white ppt. separated. The ppt. was recryst. from 95% ethanol (150 ml.), giving *N*-methyl-DL-valine (0·364 g., 17%), m.p. 240–250° (Found: N, 10·3. Calc. for $C_6H_{12}O_2N$: N, 10·7%).

α-Methyl-DL-valine has been synthesized by Kurono (1922) by hydrolysis of the amino nitrile obtained from methyl *iso*propyl ketone, but we were unable to prepare the amino acid in satisfactory yield and purity by this route. Excellent yields were, however, obtained by preparing the hydantoin instead of the amino nitrile; this method has been used recently for the synthesis of several other α-methylamino acids (Pfister *et al.* 1955).

Methyl *iso*propyl ketone (15 g.), KCN (17·2 g.) and $(NH_4)_2CO_3$ (79·2 g.) were dissolved in 50% aq. ethanol (520 ml.) and heated at 56° for 18 hr., then at 100° for

4 hr. The solution was acidified to pH 2, evaporated to 80 ml. and cooled, when *5-methyl-5-isopropylhydantoin* separated. After recrystallization from hot water, 18·05 g. (66·5%), m.p. 176–177°, was obtained (Found: N, 18·1. $C_7H_{12}O_2N_2$ requires N, 17·95%).

The hydantoin (18 g.) was boiled under reflux with $Ba(OH)_2,8H_2O$ (130 g.) in water (100 ml.) for approx. 72 hr., when no more NH_3 was evolved. The solution was filtered to remove $BaCO_3$, which was washed with hot water. The filtrate and washings were freed from remaining Ba^{2+} with CO_2 gas and $BaCO_3$ was again filtered off. The filtrate was evaporated to dryness and the residue was dissolved in hot dimethylformamide (150 ml.)–water (80 ml.). On cooling, α-methyl-DL-valine (8 g.) separated as needles, m.p. 240–260°, and by evaporation of the mother liquors to approx. 150 ml. a second crop (2·6 g., m.p. 240–260°) was obtained; the total yield was thus 10·6 g. (70%) (Found: N, 10·35. Calc. for $C_6H_{13}O_2N$: N, 10·7%).

Labelled valine. Uniformly ^{14}C-labelled L-valine was obtained from the Radiochemical Centre, Amersham. The synthesis of D- and L-[*carboxy*-^{14}C]valine and DL-[^{15}N]valine has been described elsewhere (Arnstein & Clubb, 1957).

RESULTS

Effect of cystine and its analogues on penicillin biosynthesis by washed mycelium

Of the methyl-substituted cysteine derivatives, 2·5 or 25 mM-α-methyl-DL-cystine consistently inhibited penicillin production by washed mycelium of *P. chrysogenum* WIS 51/20F3 (Table 1). This strain seems to be more sensitive than the closely related *P. chrysogenum* WIS 51/20, which was not inhibited by 7·5 mM-α-methyl-DL-cystine, but higher concentrations were not tested. Both D- and L-penicillamine disulphide (ββ-dimethylcystine)

Table 1. *Effect of cystine and cystine analogues on penicillin biosynthesis by washed mycelium of* Penicillium chrysogenum

Washed mycelium of *P. chrysogenum* WIS 51/20 F3 (0·5 g.; 0·07 g. dry wt.), harvested 58 hr. after inoculation of fermenter, was incubated for 2·5 hr. with 5 ml. of medium *A*, which also contained cystine or cystine analogues as stated.

Expt.	Compound added	Concentration (mM)	Penicillin production (i.u./ml.)	Penicillin production (% of average of control)
1	None	—	9·6, 8·5	—
	N-Methyl-L-cystine	25	9·1, 8·9	99
		2·5	8·5, 9·6	100
	S-Methyl-L-cysteine	50	8·5, 8·7	90
2	None	—	9·3, 8·1	—
	S-Methyl-L-cysteine	5	8·5, 7·4	92
	β-Methyl*threo*-DL-cystine	25	8·1, 9·3	100
		2·5	8·5, 7·4	92
	β-Methyl*erythro*-DL-cystine	25	9·1, 8·7	102
		2·5	11·75, 9·55	120
3	None	—	9·6, 9·6	—
	None (phenylacetate omitted)	—	1·2, 1·5	14
	α-Methyl-DL-cystine	25	6·6, 5·9	65
		2·5	7·1, 6·0	68
	S-Benzyl-L-cysteine	50	9·1, 8·9	94
	DL-Cystine	25	11·5, 12·0	123
4	None	—	7·8, 7·6	—
	DL-Cystine	2·5	12·8, 10·5	151
	L-Cystine	25	11·5, 10·7	144
		2·5	10·9, 10·1	136
	L-Methionine	50	7·6, 6·9	94
		5	6·9, 7·6	94
5	None	—	8·7, 8·8	—
	ββ-Dimethyl-L-cystine	25	7·4, 6·9	82
		2·5	8·1, 7·6	90
	ββ-Dimethyl-D-cystine	25	7·2, 8·1	87
		2·5	8·8, 8·9	101
6	None	—	8·1, 6·6	—
	DL-Cysteic acid	50	10·7, 9·3	135
		5	8·3, 8·3	113

also inhibited penicillin biosynthesis, but less effectively than α-methylcystine.

Stimulation of penicillin production was observed with DL-cysteic acid and with DL- or L-cystine, cystine being somewhat more potent than cysteic acid. In similar experiments with strain 51/20, DL- and L-cystine were again found to stimulate penicillin production.

No effect on penicillin biosynthesis was observed with L-methionine, *S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, *N*-methyl-L-cystine, or either isomer of β-methyl-DL-cystine (thiothreonine disulphide).

Penicillin biosynthesis in the presence of ^{14}C-labelled β-methylcystine

The benzylpenicillin *N*-ethylpiperidine salt isolated from fermentations to which ^{14}C-labelled β-methylcystine had been added was only weakly radioactive (Table 2). Since it was possible that a benzylpenicillin substituted at $C_{(5)}$ might not give an insoluble *N*-ethylpiperidine salt, the amount of ^{14}C in the supernatant solution from the precipitation of the *N*-ethylpiperidine salt and in the mother liquors from the recrystallization of the salt was also measured. From this radioactivity and the specific radioactivity of the added β-methylcystine, which is, of course, not diluted by endogenous synthesis, the maximum possible amount of penicillin derived from the ^{14}C-labelled β-methyl-DL-cystine was calculated to be less than 0·8 % of the total penicillin produced by the fermentation. It is likely, however, that these traces of radioactivity are due to an indirect utilization of isotope, for example by fixation of $^{14}CO_2$ produced by oxidation of the labelled β-methylcystine, rather than to the formation of 5-methylpenicillin.

Effect of valine and related compounds on penicillin production and valine metabolism

Penicillin biosynthesis by washed mycelium of strain WIS 51/20F3 was inhibited by α-methyl-DL-valine and, to a lesser extent, by DL-valine, whereas *N*-methyl-DL-valine had no effect (Table 3). In a similar experiment with strain WIS 51/20, DL-isoleucine was found to be a powerful inhibitor of penicillin biosynthesis, a concentration of 1 mg./ml. (approx. 7 mM) decreasing penicillin biosynthesis by approx. 50 % (Table 4).

Effect of isoleucine. A comparison of the utilization of D- and L-[*carboxy*-^{14}C]valine for penicillin biosynthesis in the presence and absence of DL-isoleucine is shown in Table 4. The addition of isoleucine resulted in a striking decrease in the uptake of D-valine by the mycelial cells, but had little or no effect on that of L-valine (Table 4, columns 4 and 5). The amount of ^{14}C present in the mycelium at the end of the experiment with D-[*carboxy*-^{14}C]valine was markedly decreased by the addition of isoleucine, whereas in the experiment with labelled L-valine the radioactivity in the mycelium was increased by about 50 % when isoleucine was added (Table 4, column 6). Although the decrease in the total amount of penicillin formed in the presence of isoleucine was similar in

Table 2. *Penicillin biosynthesis in the presence of ^{14}C-labelled β-methylcystine*

Solutions of ^{14}C-labelled β-methylcystine (see text) in dil. HCl (approx. 10 ml. of 0·2 N) were sterilized by Seitz filtration and added to the fermentation medium (1·5 l. in Expts. 1 and 3, 1 l. in Expt. 2) at various times after inoculation with spores of *P. chrysogenum* WIS 51/20, as follows: Expt. 1. 98·5, 49, 49, 55 and 59 mg. of β-methyl*threo*-DL-[*carboxy*-^{14}C]-cystine (thiothreonine *B* disulphide) were added at 45, 67, 91, 110 and 115 hr. respectively. Expt. 2. 40·1 mg. portions of β-methyl*threo*-DL-[U-^{14}C]cystine (thiothreonine *B* disulphide) were added at 41, 63, 87 and 109 hr.; Expt. 3. 100, 87, and 87 mg. of β-methyl*erythro*-DL-[*carboxy*-^{14}C]cystine (thiothreonine *A* disulphide) were added at 46, 70 and 44 hr. respectively.

		Expt. 1	Expt. 2	Expt. 3
Experimental details				
Duration of fermentation (hr.)		139	130	96
Yield of penicillin (10^{-3} i.u.)		240	249	252
Labelled amino acid added	(mg.)	310·5	165	274
	(μc)	1·14	1·02	0·81
Amount of benzylpenicillin isolated as *N*-ethylpiperidine salt	(mg.)	40	85	90
	(% of penicillin produced)	22	50	47
Comparison of specific radioactivities				
Labelled amino acid added (μc/mole*)		572	830	402
Benzylpenicillin (μc/mole)		0·56	1·62	0·18
Recovery of ^{14}C				
In penicillin produced by fermentation	(mμc)	0·23	0·69	0·08
	(%)	0·02	0·07	0·01
In mother liquor from crystallization of benzylpenicillin *N*-ethylpiperidine salt	(mμc)	0·84	0·68	<1·2
	(%)	0·07	0·07	<0·15

* Calculated for sulphydryl compound.

all flasks, the incorporation of ^{14}C from labelled D-valine into penicillin was reduced to about 20% of that taking place in the absence of isoleucine, but the conversion of labelled L-valine into penicillin was reduced comparatively little (Table 4, column 9). The effect of isoleucine on the specific radioactivity of the penicillin is, in general, similar to that on the labelling of the mycelium; i.e. a marked decrease with labelled D-valine, but an increase with labelled L-valine.

Effect of α-methylvaline. The effect of α-methyl-DL-valine on the utilization of D- and L-valine was tested with washed mycelium of *P. chrysogenum* WIS 51/20F3 (Table 5). The uptake of D-valine by

Table 3. *Penicillin production by washed mycelium in the presence of valine and analogues of valine*

In Expt. 1, washed mycelium (1 g.; 0·17 g. dry wt.) of *P. chrysogenum* WIS 51/20 F3, which had been stored at 4° for 24 hr., was suspended in 10 ml. of medium *B*, containing valine or valine analogues where stated. In Expt. 2 the experimental conditions were the same as in the experiments with cystine analogues (Table 1). In Expts. 3 and 4 medium *C* was used.

Expt.	Compound added	Final concentration (mM)	Penicillin production (i.u./ml./4 hr.)	Penicillin production (% of average of control)
1	None	—	14·1, 17·2	—
	N-Methyl-DL-valine	90	17·6, 15·1	108
		9	14·1, 11·9	86
	DL-Valine	90	11·0, 11·8	75
		9	14·8, 15·1 (i.u./ml./2·5 hr.)	99
2	None	—	9·6, 8·5	—
	N-Methyl-DL-valine	50	9·1, 8·9	99
		5	8·5, 9·6 (i.u./ml./4 hr.)	100
3	None	—	17·6, 17·6	—
	α-Methyl-DL-valine	50	13·0, 13·0	74
		5	14·0, 14·5 (i.u./ml./4 hr.)	81
4	None	—	12·9, 11·8	—
	α-Methyl-DL-valine	100	7·4, 6·8 (i.u./ml./7 hr)	58
	None	—	21·2, 21·0	—
	α-Methyl-DL-valine	100	12·2, 11·5	56

Table 4. *Effect of isoleucine on penicillin biosynthesis from* D- *and* L-*valine*

Each flask contained 4 g. of mycelium (0·6 g. dry wt.) from a 70 hr. culture of *P. chrysogenum* WIS 51/20. After a preliminary incubation for 2 hr. with 20 ml. of 3% lactose solution containing DL-isoleucine (1 mg./ml.) where indicated a further amount of 3% lactose solution (21·6 ml.) containing DL-isoleucine (21 mg., in flasks nos. 3, 4, 7 and 8 only), potassium phenylacetate (42 mg.) and either D- or L-[*carboxy*-^{14}C]valine (2 mg., 56 mμc) was added; the final concentration of DL-isoleucine was therefore approx. 7 mM, on the assumption that there was no significant catabolism of isoleucine during the pre-incubation period. After incubation for 4 hr. the penicillin content of each flask was determined by bioassay, carrier sodium benzylpenicillin (41·4 mg./flask) and DL-valine (40·1 mg./flask) were added, and penicillin and valine were isolated and assayed for ^{14}C as described in the text.

Flask no.	Labelled valine added	DL-Isoleucine present (+) or absent (−)	Uptake of valine by mycelium (mμc)	Uptake of valine by mycelium (% of added valine)	Radioactivity in mycelium (mμc)	Penicillin biosynthesis (i.u./flask)	Penicillin biosynthesis Specific radioactivity (μc/mole)	Penicillin biosynthesis Total ^{14}C in penicillin (mμc)
1	D-[*carboxy*-^{14}C]-Valine	−	24·0	42	9·5	474	524	0·422
2		−	34·0	61	12·8	443	783	0·347
3		+	16·5	29	1·8	174	54	0·016
4		+	12·4	22	5·0	193	349	0·115
5	L-[*carboxy*-^{14}C]-Valine	−	55·5	99	18·2	443	913	0·688
6		−	55·4	99	14·3	461	831	0·651
7		+	54·0	97	29·1	224	1590	0·604
8		+	54·6	98	24·3	174	1780	0·527

Table 5. *Effect of α-methylvaline on utilization of* D- *and* L-*valine for penicillin biosynthesis*

Washed mycelium of *P. chrysogenum* WIS 51/20 F3 from a 72 hr. culture was frozen slowly to −8° and stored at −70° for 57 days. Before use, it was thawed and re-washed with 0·1 M-potassium phosphate buffer (pH 6·9). Portions of mycelium (5 g.; 1·4 g. dry wt.) were suspended in 250 ml. conical flasks containing 50 ml. of medium $B+1\%$ of glucose, D- or L-[*carboxy*-^{14}C]valine (5 mg., 140 mμc) and α-methyl-DL-valine (final concentration 0·1M) where indicated. After incubation for 7 hr. at 24°, the mycelium was filtered off, washed with 0·1M-potassium phosphate buffer (pH 6·9) and water and dried at 100°. The amount of penicillin in the combined filtrate and washings was determined by bioassay, carrier sodium benzylpenicillin (50 mg./flask) and DL-valine (50 mg./flask) were added, and penicillin and valine were isolated and assayed for ^{14}C as described in the text.

Flask no.	Labelled valine added	α-Methyl-DL-valine present (+) or absent (−)	Uptake of valine by mycelium (mμc)	Uptake of valine by mycelium (% of added valine)	Radio-activity in mycelium (mμc)	Penicillin biosynthesis (i.u./flask)	Penicillin biosynthesis Specific radio-activity (μc/mole)	Penicillin biosynthesis Total ^{14}C in penicillin (mμc)
1	D-[*carboxy*-^{14}C]-Valine	−	56·8	40·6	8·0	800	78·4	0·107
2		−	99·4	71·1	13·8	904	139	0·214
3		+	49·6	35·6	6·7	516	42·8	0·038
4		+	58·2	41·6	7·1	511	46·3	0·040
5	L-[*carboxy*-^{14}C]-Valine	−	104·6	74·9	73·0	726	955	1·18
6		−	105·7	75·6	75·1	794	991	1·34
7		+	89·6	64·4	63·5	466	969	0·768
8		+	100·6	72·0	76·5	560	991	0·943

this strain was somewhat more efficient than that by strain 51/20, whereas L-valine was taken up more efficiently by mycelium of 51/20 than by 51/20F3. The result of these changes was thus a smaller difference between the uptake of L- and D-valine in the present experiment than in earlier work with strain 51/20 (Arnstein & Clubb, 1957).

The uptake of labelled L-valine by mycelium was affected only slightly by addition of α-methyl-DL-valine; that of D-valine was decreased somewhat more (Table 5, columns 4 and 5), but the effect was much less than when isoleucine was added to strain 51/20 (Table 4). The radioactivity present in the mycelium at the end of the experiments with labelled D-valine (flasks nos. 1–4) was much less than that with labelled L-valine (flasks nos. 5–8). The effect of α-methylvaline on the radioactivity of the mycelium was approximately proportional to that on the uptake of valine by the mycelial cells (Table 5, columns 4–6).

The incorporation of ^{14}C into penicillin from labelled L-valine was much greater than that from labelled D-valine in corresponding experiments (Table 5, columns 8 and 9), even when allowance is made for the less efficient uptake of labelled D-valine by the mycelium.

As in the experiments with isoleucine (Table 4), the incorporation of ^{14}C from labelled D-valine into penicillin was decreased by α-methylvaline to a much greater degree than that from labelled L-valine (Table 5, column 9). The specific radioactivity of penicillin biosynthesized from labelled L-valine was, however, not affected by addition of α-methylvaline, although this valine analogue caused a marked decrease in the radioactivity of penicillin when labelled D-valine was used as precursor (Table 5, column 8). It is noteworthy that this decrease was greater than the change in the uptake of labelled D-valine by the mycelial cells due to α-methylvaline (Table 5, columns 4 and 5).

Fig. 1 shows the effect of α-methylvaline on penicillin production by the same mycelium and under the same conditions as in the above experiment. After an initial lag period of about 2 hr., penicillin biosynthesis was inhibited uniformly throughout the remaining 5 hr. incubation. In another experiment with a different batch of mycelium essentially similar results were obtained, inhibition of penicillin production being 42% after incubation for 4 hr. and 44% after incubation for 7 hr. (Table 3).

Table 6 shows the effect of 0·1 M-α-methylvaline on the utilization of labelled valine, consisting of a mixture of L-[U-^{14}C]- and DL-[^{15}N]-valine, for the biosynthesis of penicillin and mycelial protein during incubation for 48 hr. Penicillin formation was reduced to about 40% of that in the control flasks, but the amount of ^{14}C in the crude mycelial protein and the specific radioactivity of the mycelial valine and of the penicillin was significantly increased by addition of α-methylvaline. The incorporation of isotopic nitrogen into penicillin and mycelial valine was also increased, but the dilution of ^{15}N relative to ^{14}C was not much changed by α-methylvaline: the ^{14}C:^{15}N ratios were $1·5 \pm 0·15$ and $1·7 \pm 0·2$ in penicillin, and $1·0 \pm 0·06$ and $1·2 \pm 0·03$ in the mycelial valine in the absence and presence of α-methylvaline respectively.

Because of the small amount of penicillin obtained in this experiment it was not possible to carry out a complete degradation, but it is clear from earlier work (cf. Arnstein & Clubb, 1957) that most of the ^{14}C must have been present in the penicillamine moiety. Whereas the ^{15}N content of penicillin given in Table 6 refers only to the average of both nitrogen atoms (N_4+N_8), determination of the ^{15}N content of penicillamine, isolated as the Hg mercaptide, and of penilloaldehyde 2:4-dinitrophenylhydrazone indicated that the distribution of ^{15}N between N_4 and N_8 was approx. 2:1 and was apparently not affected by addition of α-methylvaline. Accurate figures for the ^{15}N content of these degradation products could, however, not be obtained. The low ^{15}N content of the penilloaldehyde 2:4-dinitrophenylhydrazone samples (0·006–0·01 atom % excess of ^{15}N) resulted in a large percentage error in their ^{15}N analysis, and the amount of penicillamine Hg mercaptide was insufficient for further purification by converting it into *iso*propylidene penicillamine, as was done in earlier work (Arnstein & Clubb, 1957).

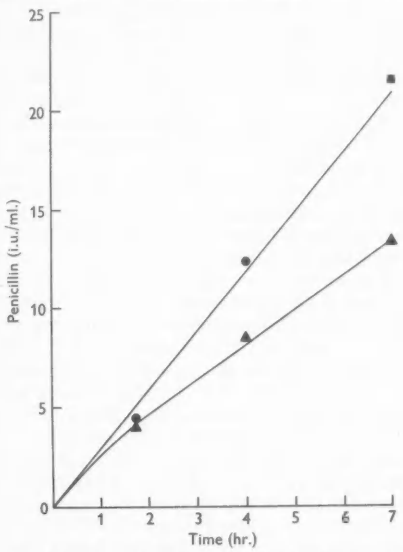

Fig. 1. Inhibition of penicillin production by α-methylvaline. Washed mycelium (2 g. portions; 0·46 g. dry wt.) of *P. chrysogenum* WIS 51/20 F3, from the same batch as used for the experiment in Table 5, was incubated with medium *C* (20 ml.) in 100 ml. conical flasks at 24°. Each point represents the mean of penicillin production in duplicate flasks in the absence (●) and in the presence (▲) of 0·1 M-α-methyl-DL-valine.

DISCUSSION

Effect of cystine and cystine analogues on penicillin biosynthesis by washed mycelium

The marked stimulation of penicillin production by washed mycelium which resulted from the addition of cystine is noteworthy because previously no consistent effect of cystine could be demonstrated (Stone & Farrell, 1946). Since the earlier work was done with complete fermentations, the biosynthesis of cystine may be rate-limiting in washed mycelium but not in a complete fermentation. Apart from cystine, only cysteic acid increased penicillin biosynthesis consistently in the present experiments. In isotope-competition experiments with $Na_2{}^{35}SO_4$ it has been shown that cysteic acid is used for penicillin synthesis in preference to sulphate, although it was a less efficient precursor than cystine (Stevens, Vohra, Moore & DeLong, 1954).

Table 6. *Utilization of valine labelled with* ^{14}C *and* ^{15}N *for penicillin biosynthesis in the presence of α-methylvaline*

Portions of fermentation medium + mycelium (50 ml.) from a 52 hr. fermentation of *P. chrysogenum* WIS 51/20 F3 were transferred aseptically into 12 sterile 250 ml. conical flasks (0·4 g. dry wt. of mycelium/flask). Where stated, 0·1 M-α-methyl-DL-valine (0·655 g./flask) was present. The flasks were incubated for 48 hr. on a rotary shaker (250 rev./min.) at 24°. At the beginning of the incubation and after 24 hr., 1 ml. of a serile solution containing L-[U-^{14}C]valine (approx. 0·15 mg.) and DL-[^{15}N]valine (0·1874 g.; radioactivity of mixture = 2·95 μc/m-mole; ^{15}N content = 15·52 atom % excess) was added to each flask. At 24 hr., 20% potassium phenylacetate (0·17 ml./flask) was also added. At the end of the incubation the mycelium was filtered off, defatted with boiling ethanol–ether (3:1, v/v), and extracted with 5% trichloroacetic acid. The specific radioactivity of the crude mycelial protein was 0·210 and 0·273 μc/g. in the absence and presence of α-methylvaline respectively. The filtrates from the appropriate flasks were combined, assayed for penicillin and carrier sodium benzylpenicillin (45·8 mg.) was added. Penicillin was isolated as the *N*-ethylpiperidine salt and assayed for ^{14}C and ^{15}N. The results are corrected for dilution by penicillin present at the beginning of the incubation and by carrier. The ^{15}N results are corrected for non-isotopic nitrogen in the *N*-ethylpiperidine salt.

Flask no.	α-Methylvaline	Penicillin production (mg. of sodium benzylpenicillin)	Isotope content of penicillin		Isotope content of mycelial valine	
			^{14}C (μc/m-mole)	^{15}N (atom % excess)	^{14}C (μc/m-mole)	^{15}N (atom % excess)
1–6	Absent	15·4	0·93 ± 0·05	0·62 ± 0·03	0·372 ± 0·015	0·369 ± 0·003
7–12	Present	6·3	1·34 ± 0·07	0·80 ± 0·05	0·524 ± 0·026	0·429 ± 0·003

The stimulation of penicillin production by cysteic acid may thus be explained by its conversion into cystine.

Penicillin biosynthesis by washed mycelium was reduced markedly by 2·5 mM or higher concentrations of α-methyl-DL-cystine, but lower concentrations were not tested. The inhibitory effect of α-methylcystine and α-methylvaline is of interest in view of the relatively limited number of examples of biologically active α-methyl amino acids (Umbreit, 1955). Inhibition of penicillin production was also obtained with ββ-dimethyl-L- and D-cystine (L- and D-penicillamine disulphide), but this cystine analogue was much less active than α-methylcystine. The inhibition of penicillin biosynthesis by penicillamine and the stimulation by cystine provide further support for the conclusion that cystine (Arnstein & Grant, 1954*a*, *b*; Stevens, Vohra, Inamine & Roholt, 1953) rather than penicillamine is the sulphur-containing precursor of penicillin.

Neither stereoisomer of β-methyl-DL-cystine had a significant effect on penicillin biosynthesis. The absence of inhibition of penicillin formation by *S*-methyl- or *S*-benzyl-L-cysteine in concentrations up to 50 mM is noteworthy because the closely related *S*-ethyl-DL-cysteine has been reported to inhibit penicillin synthesis by 23 % at mM-concentration (Demain, 1956). It is possible, however, that the activity of the latter compound was due to the D-stereoisomer.

The possible conversion of methyl-substituted cystine derivatives into penicillin

In recent experiments with tritium-labelled cystine it was found that the α-hydrogen atom and one of the β-hydrogen atoms of this amino acid were incorporated into penicillin (Arnstein & Crawhall, 1957). It seemed worth while therefore to investigate whether α- or β-methylcystine could give rise to a penicillin substituted by a methyl group at $C_{(6)}$ or $C_{(5)}$ respectively. The present experiments with both isomers of ^{14}C-labelled β-methyl-DL-cystine, as well as the earlier work with α-methyl-DL-[*carboxy*-^{14}C]cystine (Arnstein, Clubb & Grant, 1954), show, however, that such substituted penicillins are not formed to any significant extent. It is clear that at least one of the enzymes involved in the utilization of L-cystine for penicillin biosynthesis is not able to metabolize α- or β-methyl-substituted cystine analogues.

Effect of valine and related amino acids and the configuration of valine used for penicillin biosynthesis

The inhibition of penicillin biosynthesis by DL-valine and α-methyl-DL-valine confirms earlier observations by Demain (1956), who showed moreover that the effect of DL-valine was due to the D-enantiomorph. In the present work somewhat higher concentrations of these amino acids were required to produce quantitatively similar effects, but this may be ascribed to possible differences in the strains of *P. chrysogenum*, particularly since even the closely related strains WIS 51/20 and WIS 51/20F3 apparently differed with respect to inhibition by α-methyl-DL-cystine. In addition, Demain (1956) used starved mycelium which may be more susceptible to inhibition because of depletion of endogenous valine or its precursors. We have found that DL-isoleucine is also a potent inhibitor of penicillin biosynthesis, but the present results are of greater interest in connexion with the configuration of the valine used for penicillin biosynthesis.

In an earlier paper (Arnstein & Clubb, 1957) it was shown that the uptake of D-valine by the mycelium of *P. chrysogenum* WIS 51/20 was much less rapid than that of the L-enantiomorph. Moreover, this difference accounted quantitatively for the better conversion of L-valine into penicillin compared with that of D-valine, and it was suggested that the lag period in the utilization of D-valine for penicillin synthesis which was observed by Stevens, Inamine & DeLong (1956) might also be due to the inefficient uptake of this enantiomorph. With mycelium of strain WIS 51/20F3, which was used in most of the present experiments, there was much less difference between the uptake of L- and D-valine than in the previous experiments with strain WIS 51/20. Despite this more favourable uptake of D-valine, its utilization for penicillin biosynthesis relative to that of L-valine was actually less efficient. This result suggests that L-valine is used for penicillin biosynthesis in preference to D-valine, and this interpretation is supported by the inhibition experiments with both isoleucine and α-methylvaline, which were carried out with strains WIS 51/20 and WIS 51/20F3 respectively. In these experiments neither isoleucine nor α-methylvaline inhibited the uptake of L-valine by washed mycelium to any significant extent, but the uptake of D-valine was decreased by both amino acids. However, whereas the utilization of L-valine for penicillin biosynthesis, as judged by the specific radioactivity of the penicillin, was not appreciably reduced (Table 5) or even increased (Table 4) by addition of the valine analogues, the conversion of D-valine into penicillin was inhibited markedly and to a greater extent than can be accounted for by the decreased uptake of D-valine. Since the uptake of D-valine by the mycelial cells is known to be less rapid than that of the L-enantiomorph (Arnstein & Clubb, 1957), it was considered possible that the relatively

greater reduction in the conversion of D-valine into penicillin might have been due to a considerable delay in the inhibition of penicillin biosynthesis after addition of the inhibitors. If this occurred, penicillin formation would take place mainly during the early part of the incubation period, when little or no ^{14}C-labelled D-valine would have entered the cells. In the experiment with α-methylvaline this possibility has, however, been excluded by the results in Fig. 1, which show that after a relatively short lag period inhibition of penicillin is approximately constant during the remaining 70% of the incubation time. In the experiment with isoleucine, the mycelium was pre-incubated with the inhibitor, which would presumably serve to reduce the effect of any lag period, but no check was carried out on the rate of penicillin production at various times after addition of this amino acid.

The decreased utilization of D-valine for penicillin biosynthesis in the presence of α-methylvaline may be due to inhibition of D-amino acid oxidase. Inhibition of this enzyme by α-methylvaline has been reported recently (Sourkes, Heneaghe & Umbreit, unpublished experiments quoted by Umbreit, 1955). It seems possible that the effect of DL-isoleucine is due to competition of D-isoleucine with D-valine for the same D-amino acid oxidase.

These results leave little doubt that the D-configuration of the penicillamine moiety ($C_{(3)}$ of penicillin) is not derived from D-valine or from an intermediate related biosynthetically to D-valine. It is, however, not possible to decided unequivocally whether L-valine or the keto acid (α-oxo*iso*valeric acid) is the more immediate penicillin precursor, since attempts to inhibit the reversible deamination of valine were not successful, as shown by the experiment with ^{14}C,^{15}N-labelled valine, in which addition of α-methylvaline did not significantly change the relative utilization of ^{14}C and ^{15}N. The similarity in the ^{14}C:^{15}N isotope ratios of penicillamine and mycelial valine, which has been found in earlier experiments with ^{14}C,^{15}N-labelled valine (Arnstein & Clubb, 1957), suggests, however, that transamination of L-valine may be only incidental and not essential for penicillin biosynthesis.

The introduction of the D-configuration into $C_{(3)}$ of penicillin by a mechanism not involving D-valine has some resemblance to the biosynthesis of poly-D-glutamic acid by *Bacillus subtilis*. This polypeptide is apparently synthesized from L-glutamic acid without the direct participation of D-glutamic acid (Bovarnick, 1942). Although α-iminoglutaric acid has been suggested as an intermediate (Bovarnick, 1942), inversion of configuration or racemization of L-glutamic acid residues in an intermediate peptide seems equally plausible. Regardless of the detailed mechanism, the experiments on poly-D-glutamic acid, as well as the present results with penicillin, suggest that the D-configuration of amino acid residues in polypeptides and related compounds probably arises biogenetically after formation of peptide bonds rather than by utilization of free D-amino acids.

SUMMARY

1. The effect of amino acids related to cystine and valine on penicillin biosynthesis has been studied with washed mycelium of two strains of *Penicillium chrysogenum*.

2. Penicillin formation was markedly stimulated by L- or DL-cystine and by DL-cysteic acid.

3. L-Methionine, *S*-benzyl-L-cysteine, *S*-methyl-L-cysteine, *N*-methyl-L-cystine and the *erythro*- and *threo*-isomers of β-methyl-DL-cystine (thiothreonine *A* and *B* disulphides) had no effect on penicillin biosynthesis by washed mycelium. When ^{14}C-labelled β-methylcystine was added to fermentations producing penicillin, no conversion of this cystine analogue into a $C_{(5)}$-substituted penicillin could be detected.

4. Inhibition of penicillin biosynthesis was observed with α-methyl-DL-cystine and ββ-dimethyl-L- and D-cystine (L- and D-penicillamine disulphide).

5. DL-Isoleucine, α-methyl-DL-valine and DL-valine inhibited penicillin biosynthesis, but *N*-methyl-DL-valine had no effect.

6. Both isoleucine and α-methylvaline inhibited the conversion of ^{14}C-labelled D-valine into penicillin to a greater extent than that of L-valine.

7. A different strain of *Penicillium chrysogenum* (WIS 51/10F3) from that used in previous work utilized L-valine for penicillin biosynthesis much better than D-valine.

8. It is concluded from the better conversion of L-valine into penicillin by strain WIS 51/20F3 and from the preferential inhibition of the utilization of D-valine by α-methylvaline or isoleucine that D-valine is not a penicillin precursor, but that either L-valine or, less likely, the keto acid, α-oxo*iso*valeric acid, is the precursor of the penicillamine moiety of penicillin.

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The Amino Acid Compositions of Three Fractions from Oxidized Wool

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The complex histology of the wool fibre and its insolubility in all solvents are the main obstacles to the elucidation of its chemical structure. On histological grounds alone it is apparent that wool is not a homogeneous protein, and therefore the logical approach to the problem would be to isolate the different structural components and study them separately. Unfortunately, in our opinion, it is not possible to do this at present since it is by no means clear how many components there are in the wool fibre or how they can be separated in a pure state. Most of the chemical methods used in their isolation result in considerable degradation of material, and in some cases several components are inextricably mixed. Moreover, histologists differ about the exact sites in the fibre structure which the isolated components originally occupied, and there is some confusion in the naming of them. A good review of the histological findings has been given by Mercer (1953), and further reference should be made to the *Proceedings of the International Wool Textile Conference*.

In view of the foregoing therefore most of the past and present research into the chemical structure of wool has been, and is being, directed towards the dissolution of the whole wool fibre and the isolation of large, homogeneous peptides from the resultant solutions. Clearly such procedures are only second best, as the assignment of any given peptide to a histological component of the fibre cannot be free from doubt. Two main methods of dissolution have been used, and in both the essential preliminary degradative reaction has been the fission of the disulphide linkages. Gillespie & Lennox (1953) used alkaline thioglycollate solutions to reduce the disulphide linkages and to dissolve the wool, and Alexander & Hudson (1954) oxidized these bonds with peracetic acid and dissolved the oxidized wool in dilute ammonia solution. The latter method is analogous to that used by Sanger (1949) for the separation of the *A* and *B* chains of insulin, and has been adopted in the present work. Alexander & Hudson (1954) obtained three main fractions from oxidized wool which they named α- β- and γ-keratoses. In our opinion it is doubtful whether the fractions are sufficiently well characterized to warrant the term 'keratoses', but we have retained this nomenclature because other workers have already made reports on their constitutions (Earland & Knight, 1955, 1956), their physical properties (Alexander, 1951; O'Donnell & Woods, 1955) and on the amounts of their *N*-terminal amino acids (Alexander & Smith, 1955). This paper describes the

preparation of the α-, β- and γ-keratoses from an Australian Merino 64s quality wool (Corfield & Robson, 1955) and gives their amino acid compositions. The work is a necessary preliminary to a more detailed study of the chemical structure and physical properties of these fractions which is being undertaken in these laboratories.

EXPERIMENTAL

Oxidation of wool with peracetic acid. The following procedure was used to oxidize all the wool used in the investigation, amounts being varied proportionately as required; and the term 'oxidized wool' as used hereafter refers to wool treated in this way. An amount (1 g. dry wt.) of wool was stirred continuously for 24 hr. at 25° with 100 ml. of 1·6% (w/v) peracetic acid, filtered and washed with 1 l. of water. Total N determinations on the combined washings and filtrate showed that 1% of the total N of the wool dissolved. Cystine determinations, by the method of Shinohara (1935), showed that less than 1% of the disulphide linkages remained unoxidized.

Preparation of α-, β- and γ-keratoses. Preliminary work showed that varying amounts of oxidized wool could be dissolved by solutions of NaH_2PO_4, Na_2HPO_4, Na_3PO_4 and NH_3 according to their concentrations and the time of extraction at room temperature, but in no experiment was the residue less than 10% of the original wool. Gradient extraction by 0·2M-phosphate solution over the range pH 4·5–11·5 resulted in the separation of two distinct fractions that dissolved in the pH ranges 4·5–7 and 9–11·5. The former represented 29% of the total N of the oxidized wool and was soluble at all pH values, whereas the latter furnished 56% of the total N and could be precipitated at pH 5. Similar yields of these fractions could be obtained by extracting oxidized wool at 20° for 24 hr. with 0·2M-Na_3PO_4, acidifying to pH 5 with H_3PO_4 and estimating the total N in the resultant precipitate (α-keratose) and filtrate (γ-keratose). The difficulty in removing the phosphate from the soluble fraction, however, led us to abandon the procedure in favour of that outlined in Fig. 1. The 200 g. of oxidized wool used was prepared from the sample of Australian Merino 64s quality wool previously analysed by Corfield & Robson (1955).

The α- and γ-keratose preparations were obtained as dry, white powders, whereas examination of the β-keratose preparation showed that it retained certain fibre characteristics. The $\frac{3}{32}$ in. pieces of wool fibre resembled curved hollow tubes, indicating that most of the dissolved wool was derived from the wool cortex and that the β-keratose was probably mainly cuticular. Alexander & Earland (1950) in fact describe it as subcuticle membrane, but this statement has been challenged by Mercer (1953).

Hydrolysis procedure. All hydrolysates were prepared by refluxing approx. 0·4 g. of material with 20 ml. of 5·7N-HCl for 24 hr., removing HCl *in vacuo* and making the residues up to 10 ml. with water.

Chromatographic separations and estimations of amino acids. The basic amino acids were separated from 20 mg. of hydrolysate according to the directions of Moore & Stein (1951), and the other amino acids were separated from 5 mg. of hydrolysate by elution chromatography on 0·9 cm. × 100 cm. columns of the ion-exchange resin Zeo-Karb 225 (8×). Amino acid estimations on the eluent fractions were made by the photometric ninhydrin procedure of Moore & Stein (1948).

Total nitrogen, total sulphur and ammonia. Total N contents of α-, β- and γ-keratoses and of oxidized wool were determined (Chibnall, Rees & Williams, 1943). The NH_3 contents of the hydrolysates were determined as described previously (Corfield & Robson, 1955). The total S contents of the four samples were determined by the Benedict–Denis method (Barritt, 1934).

RESULTS

Amino acid analyses of the α-, β- and γ-keratose preparations are given in Table 1. The results are the means of triplicate determinations, and no corrections have been applied for amino acids destroyed during hydrolysis or chromatography. There were no unidentified peaks on the chromatograms, but the cystine and methionine peaks were missing; the former was presumably oxidized to cysteic acid and the latter to methionine sulphone. Cysteic acid was estimated during chromatography, but methionine sulphone did not emerge as a separate peak. However, methionine is only present to the extent of 0·56% in wool, and its omission cannot materially affect the overall analysis. No tryptophan determinations were made. Since the relation between the total S of proteins and their estimated content of S-containing amino acids is always of interest, the total S contents of all the preparations were determined.

In addition to these results, Table 1 contains the incomplete amino acid analyses of α-keratose and γ-keratose fractions prepared from an oxidized sample of Australian Merinos 56s quality wool tops. The γ-keratose preparation was isolated by the procedure outlined in Fig. 1, and the α-keratose by the second phosphate-isolation method described above in the preliminary experimental work. The results are given to only two significant figures as they represent only one analysis of each keratose, and the glycine and alanine peaks were not resolved sufficiently well for determinations of their contents to be made. On the assumption that the percentage yields of α-, β- and γ-keratoses from oxidized wool are approx. 60, 10 and 30 respectively as claimed by Alexander & Hudson (1954) and substantiated by the present work, the figures in Table 2 have been derived from the results given in Table 1. This procedure serves as a check on the overall analysis.

DISCUSSION

The results presented in Table 1 show several interesting features. In the first place, the amino acid composition of oxidized wool is in close agreement with that of the virgin wool (Corfield & Robson, 1955) from which it was prepared, and for

Table 1. *Amino acid analyses of hydrolysates of oxidized wool, α-, β- and γ-keratoses*

	Total N (%)						
			Fractions from oxidized wool				Australian Merino 64s quality wool (Corfield & Robson, 1955)
Amino acid	γ-Keratose from wool tops (see text)	α-Keratose from wool tops (see text)	γ-Keratose	α-Keratose	β-Keratose	Oxidized wool before fractionation	
Alanine	—	—	2·58	4·83	4·84	3·99	4·12
Ammonia	—	—	11·05	10·25	8·61	9·34	—
Arginine	21·0	20·0	19·0	20·8	17·7	19·2	19·1
Aspartic acid	1·6	6·8	1·79	6·25	4·75	4·56	4·38
Cysteic acid	19·0	3·5	14·5	3·72	4·40	6·50	—
Glutamic acid	6·7	12·0	5·87	10·9	7·32	8·50	8·48
Glycine	—	—	4·97	5·16	6·53	6·09	6·29
Histidine	1·6	1·6	1·57	1·24	2·64	1·68	1·91
Isoleucine	2·3	2·7	2·14	2·49	2·85	2·34	2·44
Leucine	2·3	7·8	2·55	7·30	6·17	5·78	5·85
Lysine	1·3	4·8	1·03	4·60	6·43	4·02	3·92
Phenylalanine	0·9	1·9	1·15	1·94	2·35	1·90	2·07
Proline	11·0	2·1	9·85	2·69	4·57	4·89	5·05
Serine	11·5	6·8	9·70	6·70	7·99	7·50	7·87
Threonine	7·9	3·6	7·46	3·45	4·40	4·66	4·70
Tyrosine	1·4	2·7	1·41	2·44	2·25	2·37	2·62
Valine	5·0	3·9	4·15	3·98	4·41	3·97	4·16
Total	—	—	100·77	98·74	98·21	97·29	—
Total N (%)	13·2	15·9	13·5	16·0	15·6	15·7	16·3
Total S (%)	—	—	5·84	1·88	2·13	3·12	3·38

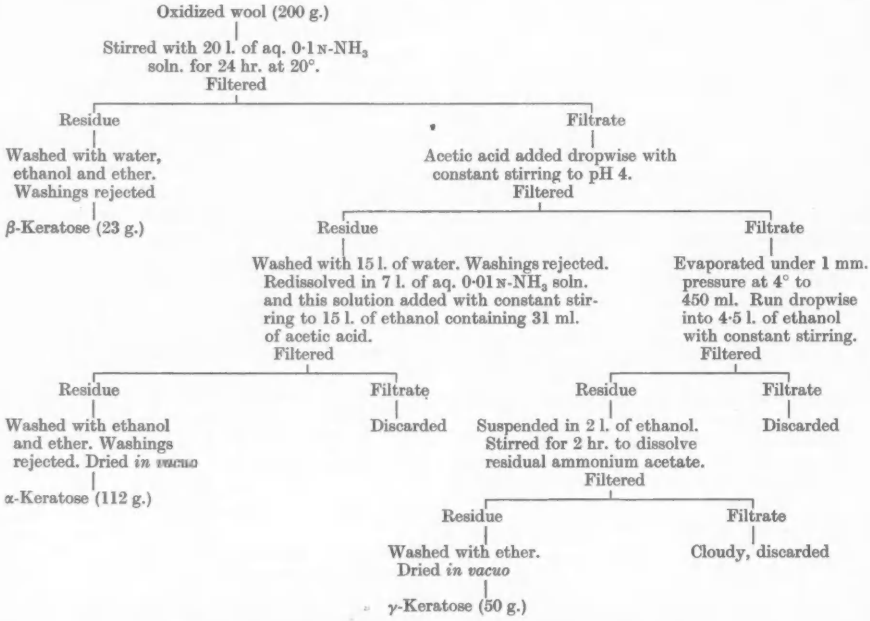
Oxidized wool (200 g.)
Stirred with 20 l. of aq. 0·1 N-NH3 soln. for 24 hr. at 20°.
Filtered
Residue
Washed with water, ethanol and ether. Washings rejected.
β-Keratose (23 g.)
Filtrate
Acetic acid added dropwise with constant stirring to pH 4.
Filtered
Residue
Washed with 15 l. of water. Washings rejected. Redissolved in 7 l. of aq. 0·01 N-NH3 soln. and this solution added with constant stirring to 15 l. of ethanol containing 31 ml. of acetic acid.
Filtered
Residue
Washed with ethanol and ether. Washings rejected. Dried in vacuo
α-Keratose (112 g.)
Filtrate
Discarded
Filtrate
Evaporated under 1 mm. pressure at 4° to 450 ml. Run dropwise into 4·5 l. of ethanol with constant stirring.
Filtered
Residue
Suspended in 2 l. of ethanol. Stirred for 2 hr. to dissolve residual ammonium acetate.
Filtered
Residue
Washed with ether. Dried in vacuo
γ-Keratose (50 g.)
Filtrate
Cloudy, discarded
Filtrate
Discarded

Fig. 1. Preparation of α-, β- and γ-keratoses from oxidized wool.

only three amino acids do the analyses differ by more than 5%. The amounts of histidine, phenylalanine and tyrosine are respectively 14, 6 and 11% less in oxidized wool than in virgin wool. The use of peracetic and performic acids to oxidize the cystine of proteins to cysteic acid has been reported partially to destroy other amino acids according to the conditions of reaction (Toennies & Homiller, 1942; Hirs, 1954). With particular reference to wool, Simmonds (1955) has shown that performic acid oxidation of a keratin derivative extracted from wool caused significant losses of serine, threonine, tyrosine, phenylalanine and histidine, and it is notable that the last three of these suffered partial loss during the peracetic acid oxidation of wool in the present work. It should also be borne in mind that 1% of the total nitrogen of virgin wool went into solution during this process.

The differences in amino acid compositions of the three keratoses are quite striking. γ-Keratose contains much larger amounts of cysteic acid, proline, serine and threonine, and much smaller amounts of alanine, aspartic acid, glutamic acid, leucine, lysine, phenylalanine and tyrosine than does oxidized wool, whereas the reverse is true of α-keratose. It is perhaps surprising to find that β-keratose has an amino acid composition roughly similar to that of wool since it is said to be a mixture of cortical-cell membranes and resistant cuticle material (Mercer, 1953) comprising only 10% by weight of wool. However, its phenylalanine, glycine, lysine, valine and histidine contents are the greatest of all four preparations, whereas its arginine content is the least. A rough check on the detailed analyses given in Table 1 is furnished by the results in Table 2, which show that the composition of oxidized wool can be quantitatively accounted for by summing the amino acid compositions of the three keratoses in the proportions stated, with the exceptions of the glycine, histidine, lysine, phenylalanine and tyrosine totals, which are all low. No satisfactory explanation can be offered for these discrepancies.

The total nitrogen and total sulphur contents of the three keratoses are compared in Table 3 with the results of previous workers. There is fair agreement for the total nitrogen contents of α- and β-keratoses, but the figures for the total sulphur contents show wide differences. The total sulphur contents of β- and γ-keratoses, as determined by Alexander & Smith (1955), agree well with those found in the present work, but their figure for α-keratose is much higher. Earland & Knight (1955) claim that α- and β-keratoses contain much more sulphur than was found either in the present work or in that of Alexander & Smith. It is considered that their figures are probably too high, for if the total sulphur content of γ-keratose is taken as 6%, the total sulphur content of their wool would be 3·8%, which is much greater than is usually found in wool. When considering the total sulphur contents of the α-, β- and γ-keratoses and of oxidized wool obtained in the present work, it is interesting to note that their cysteic acid contents account for only 72·3, 73·6, 76·8 and 74·5% of them respectively. These results are in accord with the finding of Corfield & Robson (1955) that the cystine content of wool could account for only 80% of its total sulphur.

Table 2. *Amino acid composition of a mixture of 60% α-keratose, 10% β-keratose and 30% γ-keratose compared with that of oxidized wool*

Amino acid	Nitrogen (g.) from 600 g. of α-keratose + 100 g. of β-keratose + 300 g. of γ-keratose	Nitrogen (g.) from 1 kg. of oxidized wool
Alanine	6·44	6·26
Ammonia	15·7	14·6
Arginine	30·4	30·1
Aspartic acid	7·46	7·16
Cysteic acid	10·1	10·2
Glutamic acid	14·0	13·4
Glycine	8·01	9·56
Histidine	2·24	2·64
Isoleucine	3·70	3·67
Leucine	9·00	9·08
Lysine	5·83	6·30
Phenylalanine	2·67	2·98
Proline	7·27	7·68
Serine	11·6	11·8
Threonine	7·02	7·32
Tyrosine	3·26	3·72
Valine	6·19	6·23
Total N (%)	15·2	15·7
Total S (%)	3·09	3·12

Table 3. *Total nitrogen and total sulphur analyses of α-, β- and γ-keratoses by various authors*

	Total N (%)			Total S (%)		
Material	Earland & Knight (1955)	Alexander & Smith (1955)	Present work	Earland & Knight (1955)	Alexander & Smith (1955)	Present work
α-Keratose	16·11	15·95	16·0	2·90	2·5	1·88
β-Keratose	15·94	15·40	15·6	2·72	2·2	2·13
γ-Keratose	—	—	13·5		6·13	5·84

Finally, it is interesting to compare the amino acid analyses of the α- and γ-keratoses prepared from virgin wool of 64s quality with those of the corresponding wool fractions obtained from wool tops of 56s quality. Reference to Table 1 shows that the agreement between the two sets of results is quite close, and it would appear that the two keratoses can in fact be characterized by their amino acid compositions. Also, with α-keratose, neither a change in the degradation procedure adopted for its preparation nor a change in the quality of the wool used has a marked effect on its amino acid composition.

SUMMARY

1. An Australian Merino 64s quality wool has been oxidized with peracetic acid and its amino acid composition determined. The analyses is in excellent agreement with that of virgin wool (Corfield & Robson, 1955), except that cystine is converted into cysteic acid and that small losses of histidine, tyrosine and phenylalanine occurred during oxidation.

2. α-, β- and γ-Keratoses were prepared from oxidized wool and their amino acid compositions determined.

3. Only 75% of the total sulphur of oxidized wool and of the three keratoses could be accounted for by their respective cysteic acid contents.

4. The total nitrogen and total sulphur of the three keratoses have been compared with the amounts determined by other workers. For total sulphur the agreement is not good.

We are indebted to Dr F. O. Howitt for many helpful discussions during the course of this work, and to Miss E. M. Booth for carrying out the sulphur analyses.

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The Identification of Two Lipid Components in Liver which enable *Penicillium notatum* Extracts to Hydrolyse Lecithin

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Fairbairn (1948) reported that aqueous dialysed extracts of the mould *Penicillium notatum* possessed an active phospholipase *B* which attacked the lysophosphoglycerides but had no action on the intact phosphoglycerides. The same preparation has been used as the basis of an enzymic method for the preparation of glycerylphosphorylcholine (Uziel & Hanahan, 1956) and here again no activity was found towards intact lecithin. Dawson (1957) attempted to detect lysolecithin in the crude lipids extracted from liver with a similar enzyme preparation from *P. notatum*, but found that, although the mould extract did not attack purified ovolecithin, there was a rapid and almost quantitative release of the fatty acids from the lecithin present in the crude lipids extracted from liver. This activity was not caused by some difference between the ovolecithin and its counterpart in the liver lipids, since when purified the latter lost its susceptibility to attack by the *P. notatum* extracts. It became apparent that there was an additional factor in the crude lipid extract which was responsible for the *P. notatum* phospholipase *B* preparation attacking the liver lecithin. This paper

describes the isolation and identification of two lipids from the crude liver lipids, both of which, when added at low concentrations to a system containing purified lecithin and *P. notatum* phospholipase *B*, will bring about a degradation of the phosphoglyceride into glycerylphosphorylcholine and free fatty acids.

EXPERIMENTAL

Preparation of P. notatum *extracts.* The method of mould culture and the extraction of enzyme closely followed that of Fairbairn (1948). The *P. notatum* used was strain 1249/B/21, and was maintained on a Czapek agar slope. It was surface cultured at 25° on a corn-steep-liquor medium, and the mat of green sporulating mycelium which grew in about 12 days was separated from the medium by tipping the entire culture through a Büchner funnel. The mycelium was then washed with a little water, and damp-dried by pressing between filter paper. It was then ground to a slurry in a mortar with water (5 ml./g. of wet mycelium) and autolysed for 24 hr. at 25° in the presence of a little toluene. The avoidance of drying the mycelium before autolysis gave a better final yield of the enzyme. The autolysate was filtered and the golden-brown filtrate was dialysed against water at 4° for 24 hr. The dialysis sac was immersed in glycerol and removed when the volume of the contents had decreased to about one-quarter. The glycerol extract obtained had potent phospholipase *B* activity, but contained little protein; it could be stored for over a year at 0° without noticeable loss of activity.

For some experiments the enzyme was partially purified by fractionation with $(NH_4)_2SO_4$. The aqueous dialysed extract of the mould was mixed with 0·05 vol. of acetate buffer, pH 4·5. The mixture was cooled to 0° and solid $(NH_4)_2SO_4$ was added (50 g./100 ml.). The precipitate of inactive material was centrifuged off and discarded, and the supernatant containing the enzyme was, after filtration, dialysed until free of $(NH_4)_2SO_4$.

Materials. The methods used for the preparation of lecithin, lysolecithin and glycerylphosphorylcholine are given by Dawson, Mann & White (1957).

Estimations

Inositol. This was assayed by the microbiological procedure of Campling & Nixon (1954), the *Kloeckera brevis* yeast being kindly supplied by Dr E. F. Hartree. Samples of lipid were hydrolysed before inositol assay by heating at 105° for 18 hr. in 5N-HCl: the hydrolysate was evaporated to dryness *in vacuo* and the residue dissolved in water and adjusted to pH 5 before analysis. Samples were assayed in triplicate and standard growth curves (0–5 μg. of inositol) were prepared for each set of determinations. After 3 days' growth, turbidities were read in an EEL colorimeter with an OR1 red filter.

Glycerol. The method of estimation was largely based on an unpublished method kindly supplied by Dr J. N. Hawthorne except for the periodate reaction and titration, which was a micro-modification of the method of Voris, Ellis & Maynard (1940). Samples were hydrolysed as for inositol assay, and then portions were run on paper chromatograms (acetic acid-washed Whatman no. 1) with propan-2-ol–acetic acid–water (3:1:1, by vol.). In other lanes were run standard glycerol spots and blanks and, on either side of the chromatogram, glycerol markers which were located by the $AgNO_3$–alkali method of Trevelyan, Procter & Harrison (1950). Areas corresponding to the standard and unknown glycerol spots were cut out and eluted with water. The combined aqueous extracts (12 ml.) of a particular spot were mixed with 1 ml. of a solution containing 0·125 g. of KIO_4 in 500 ml. of 0·02N-H_2SO_4 and kept for 1 hr. at room temp. A volume (0·1 ml.) of $MgSO_4,7H_2O$ soln. (15%, w/v) was added and then dil. NaOH soln. until a very faint turbidity developed. This was just cleared by the addition of 0·02N-H_2SO_4 and the solution was mixed with 3 ml. of phosphate buffer (12 g. of $Na_2HPO_4,2H_2O$ and 42 ml. of N-H_2SO_4 diluted to 100 ml.). Then 0·2 ml. of 5% (w/v) KI soln. was added and the liberated I_2 was titrated with freshly diluted 0·001N-$Na_2S_2O_3$ soln., recently prepared starch solution being used as indicator. Standard glycerol curves were set up for each determination and the unknown was read directly from them; they were straight lines cutting the axis at about 1–2μg. of glycerol.

Other estimations. Glycerylphosphorylcholine and free fatty acids were determined as described by Dawson (1956), acyl fatty-ester bonds by the method of Stern & Shapiro (1953) and phosphorus by the method of Fiske & Subbarow (1925). Aldehyde assays were kindly performed by Dr D. N. Rhodes by the method of Wittenberg, Korey & Swenson (1956). Nitrogen was estimated by a Conway microdiffusion technique after digestion overnight with the reagent of Borsook & Dubnoff (1939).

Chromatographic detection of glycerylphosphoryl 'base' was carried out as described by Dawson (1955*a*).

Isolation of active lipids

The source of active lipids mostly used was rat or sheep liver; recently horse liver has provided a rich source of one of the active lipids (monophosphoinositide).

Separation of methanol-soluble lipids. Fresh liver tissue (20 g.) was blended in a high-speed blender with 160 ml. of $CHCl_3$–methanol (1:1, v/v). The suspension was centrifuged and the supernatant evaporated to dryness *in vacuo*; the residue was extracted with 0·5 vol. of methanol for every volume of supernatant used. The precipitate of methanol-insoluble lipids was removed by centrifuging and discarded; it possessed little activity.

Chromatography on activated aluminium oxide. The solution of methanol-soluble lipids (50 ml.) was evaporated to dryness *in vacuo*. The residue was taken up in 25 ml. of $CHCl_3$–methanol (1:1, v/v), any undissolved material being centrifuged off and discarded. The dissolved lipids were then applied equally on two activated alumina (Hopkin and Williams Co. Ltd.) columns, 1·8 cm. diam., 22 cm. long; $CHCl_3$–methanol was passed through the columns until 150 ml. was collected from each (30 min.). This effluent contained the lecithin in the lipid and much neutral fat and cholesterol but virtually no activity. The activity was eluted from the column by $CHCl_3$–ethanol–water (2:5:2, by vol.) (Rhodes & Lea, 1957). It can be seen (Fig. 1) that the phosphorus from the column appears a little before the activity and consequently an enrichment was obtained by discarding the first 125 ml. of solvent coming through each column and collecting the next 125 ml. No improvement of this separation was obtained

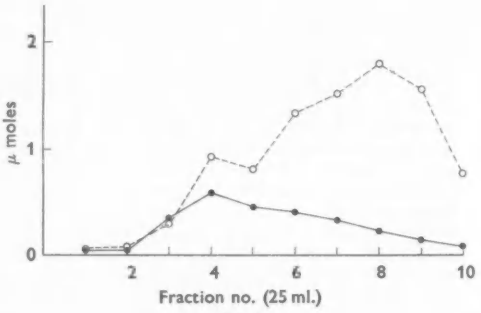

Fig. 1

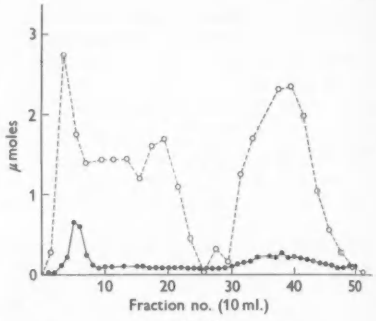

Fig. 2

Fig. 1. Elution of activity from an alumina column with $CHCl_3$–ethanol–water (2:5:2, by vol.). ○, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system (i.e. 3·5 μmoles of liver lecithin in 1 ml. of acetate buffer, pH 4, incubated with 0·2 ml. of *P. notatum* enzyme for 6 hr. at 37°); ●, phosphorus contained in 1 ml. of fraction.

Fig. 2. Fractionation of activity on a short silicic acid column (11 cm.) with $CHCl_3$–methanol (4:1, v/v). ○, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system as in Fig. 1; ●, phosphorus contained in 1 ml. of fraction.

Fig. 3. Fractionation of activity on a long silicic acid column (28 cm.) with $CHCl_3$–methanol (4:1, v/v). ○, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system as in Fig. 1, except that lecithin was 1·75 μmoles; ●, phosphorus contained in 1 ml. of fraction.

by lengthening the column, varying the solvent or increasing the time usually taken for the elution of activity with the second solvent (90 min.).

Chromatography on an activated silicic acid column. The active lipids were next chromatographed on a column of activated silicic acid (Mallinckrodt Ltd.) essentially as described by Lea, Rhodes & Stoll (1955). The combined active fractions from the alumina columns (250 ml.) were evaporated to dryness *in vacuo*. The residue was dissolved in 15 ml. of $CHCl_3$–methanol (4:1, v/v) and centrifuged; the supernatant was applied to a silicic acid column (1·8 cm. diam.; 11 cm. long). The $CHCl_3$–methanol solvent (4:1, v/v) was used to develop the column and 10 ml. fractions of the effluent were collected in an automatic fraction collector. Two main peaks of activity consistently appeared (Fig. 2): the first, in fractions 2–5 and just before the main phosphorus peak which was identified as phosphatidylethanolamine, and the second a long peak between fractions 30 and 45, which was coincident with a phosphorus peak at the same position (I). In some preparations, activity was also observed between these two peaks (Fig. 2), but in others it was completely absent. Fractions 1–10 were now combined and run in the same solvent on a long silicic acid column (28 cm.); this enabled a clear separation to be made of a minute phosphorus peak (II) containing the bulk of the activity and the major phosphatidylethanolamine peak (Fig. 3). The phosphatidylethanolamine usually possessed some activity in the test system, but this was very much lower on a molar basis than the activity of the initial peak, and was not observed at all with low concentrations of the phosphoglyceride.

The fractions comprising silicic acid peak I were evaporated to dryness *in vacuo*, the lipid was dissolved in 2 ml. of hot ethanol and the solution cooled for 1 hr. at 0°. The lipid precipitate consisted of minute transparent spheroids which tended to aggregate in clusters (active lipid I). The minute phosphorus-containing peak II obtained from the long silicic acid column was dissolved in 1 ml. of ether; it was slightly yellow and showed fluorescence in ultraviolet light. Acetone (3 ml.) was added and the ether removed by evaporation under reduced pressure. A white precipitate formed which possessed most of the phosphorus and activity, the yellow fluorescence being left in solution. The precipitate was collected by centrifuging and was washed with acetone (active lipid II).

RESULTS

Breakdown of liver phospholipids by P. notatum *extracts*

In initial experiments emulsions of crude liver lipids were incubated with the *P. notatum* phospholipase *B* preparation and the liberation of glycerylphos-

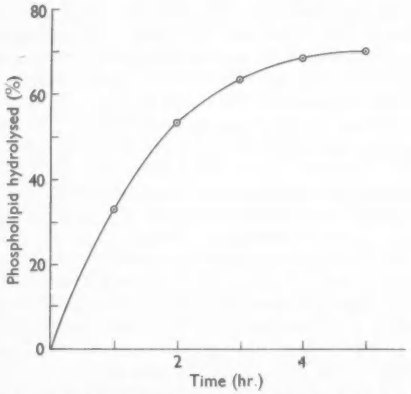

Fig. 4. Breakdown of rat-liver phospholipids on incubation with a *P. notatum* phospholipase *B* preparation. Chloroform–methanol-soluble phospholipids from 110 mg. of fresh liver were incubated at 37° with 0·2 ml. of *P. notatum* extract in acetate buffer, pH 4.

phorylcholine was measured, with the expectation that this would indicate the level of lysolecithin present in the lipids. However, the release of glycerylphosphorylcholine, measured as water-soluble acid-labile choline, was far in excess of the amount of lysolecithin which could reasonably be expected to be present in the tissue. Furthermore, when a liver-lipid emulsion was incubated with *P. notatum* extract at pH 4, a distinct physical change occurred: the originally milky fluid coalesced and a layer of fatty substance separated, leaving a clear underlying layer of the suspending medium. A considerable portion of the phosphorus in the lipid became water-soluble and was no longer precipitable by perchloric acid (Fig. 4). Moreover, a considerable release of free fatty acids occurred in the mixture and its acyl-fatty acid ester content decreased. All of the water-soluble choline which appeared in the reaction mixture was combined in an acid-labile form and no free base was liberated. By paper chromatography, after removal of the glycerol from the enzyme preparation by dialysis, it was confirmed that not only glycerylphosphorylcholine but also glycerylphosphorylethanolamine had been released. These observations strongly suggested that the main reaction occurring in the mixture was the breakdown of the intact phosphoglycerides, lecithin and phosphatidylethanolamine into free fatty acids and the corresponding glycerylphosphoryl 'bases'.

The liver phosphoglycerides were not attacked because of any structural peculiarity, e.g. in their fatty acid composition. When, for example, egg lecithin, which by itself was not attacked by the enzyme preparation, was added to the liver lipids

Table 1. *Breakdown of lecithin by* P. notatum *extracts*

System contained 3·3 μmoles of ovolecithin (Expt. 1) or of liver lecithin (Expt. 2) in 1 ml. of acetate buffer, pH 4, and 0·2 ml. of *P. notatum* extract. Incubated for 340 min. at 37°.

Expt. no.	Active lipid added (μmole P)	Liberation of glycerylphosphorylcholine (μmole)
1	0	0
Active lipid I (monophosphoinositide)	0·042	0·096
	0·164	0·440
	0·290	0·955
2	0	0
Active lipid II (polyglycerol phospholipid)	0·052	0·27
	0·075	0·55
	0·090	0·75

there was a marked stimulation of the reaction. Furthermore, when liver lecithin was separated from the other phospholipids by an alumina-column technique (Hanahan, 1954), it was no longer susceptible to attack by the enzyme preparation. The simplest explanation, therefore, was that in the lipid extract itself there was an activating compound which in some way assisted the enzyme preparation to attack whole phosphoglyceride molecules. It is well known that crude lipid extracts impart a lipid-solvent solubility on to non-lipids which are therefore carried along with the lipids themselves. However, when a liver lipid extract was purified from such non-lipids by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951), the breakdown of phosphoglycerides on adding *P. notatum* enzyme still occurred at the same rate. The active agent was precipitated from ethereal solution by acetone, which suggested that it might be associated with the phospholipid fraction. It was stable when suspended in an aqueous buffer (pH 4) and kept at 100° or even after a brief autoclaving at 120°, but 5 min. in 0·1 N-HCl at 100° or in methanolic 0·2 M-KOH at room temperature was sufficient to suppress virtually all activity.

Identification of active lipids

The active lipids, I and II, isolated as described in the Experimental section caused the liberation of glycerylphosphorylcholine when added to a system containing pure lecithin, *P. notatum* phospholipase *B* and acetate buffer at pH 4 (experiments reported in Table 1 are typical), effects still being demonstrable when the lipids were added individually at concentrations as low as 30 μM, 1 phosphorus atom/molecule being assumed to be present. The molar release of glycerylphosphorylcholine was very many times

greater than the molar amount of active lipid which was added to the system.

Lipid I. The lipid contained phosphorus but no nitrogen. After acid hydrolysis in 5N-HCl at 100°, followed by paper chromatography in propan-2-ol–acetic acid, two reducing substances became visible on spraying with alkaline silver nitrate; they were identified as glycerol and inositol. The lipid gave no Molisch test for carbohydrate (Found: inositol, 22·7; P, 3·85%; molar ratio inositol: P:fatty acid:glycerol, 1·0:1·0:2·0:0·9. Calc. for dipalmitoylglycerylphosphorylinositol: inositol, 22·5; P, 3·8%; molar ratio, 1:1:2:1). The lipid was therefore a monophosphoinositide.

Lipid II. This lipid was isolated only in very small amounts; thus from each preparation from rat liver only about 40–60 μg. of P was obtained. The N:P molar ratio was $<0{\cdot}07:1$. On acid hydrolysis, followed by paper chromatography of the hydrolysate in propan-2-ol–acetic acid, only a single reducing spot corresponding to glycerol became visible on spraying with alkaline silver nitrate. No inositol was detected either by paper chromatography or by the microbiological method, nor was carbohydrate found by the Molisch test. The molar ratio fatty acid:P:glycerol was 2·8:2·0:2·9. On methanolic alkaline hydrolysis followed by paper chromatography, no glycerophosphoric acid was detectable as would be expected if the lipid was a simple phosphatidic acid (Dawson, 1954). The alkaline hydrolysis product also contained a little aldehyde. McKibbin & Taylor (1952) reported the isolation from horse liver of a polyglycerol phospholipid which approximated to a molar ratio of fatty acid:P:glycerol of 3:2:3. The observations reported here suggest that active lipid II is a compound of this type, which possibly contains in addition a nitrogen-free lipid containing a long-chain fatty aldehyde.

DISCUSSION

Two phospholipids, monophosphoinositide and polyglycerol phospholipid, have been isolated from liver and when these are added at low concentrations to a system containing lecithin and *P. notatum* phospholipase *B* they enable the enzyme to attack the intact lecithin molecules. This is a novel type of activation of an enzyme system and, if it is assumed that the lecithin is degraded to glycerylphosphorylcholine by a successive enzymic removal of the two fatty acids (i.e. via lysolecithin), as the preparation is capable of attacking lysolecithin very rapidly the net effect of the activation is the development of phospholipase *A* activity in the system for removing the first fatty acid. If this is so, the effect could be considered either as the addition of an essential cofactor which activates a latent phospholipase *A* in the enzyme preparation or a changing of the specificity of the phospholipase *B*. However, other explanations are possible and work is in progress with a view to ascertaining the precise mechanism of the activation.

It is by no means certain that these two lipids represent the only ones that are active and indeed some evidence has been obtained that phosphatidylethanolamine can in much higher concentrations cause a small breakdown of liver lecithin by the *P. notatum* extracts.

Monophosphoinositide was first isolated from animal tissues by Faure & Morelec-Coulon (1954), using heart muscle. McKibbin (1954), Hawthorne (1955) and Dawson (1955*b*) have described the presence of lipid inositol in liver, and McKibbin (1956) isolated monophosphoinositide contaminated with a little nitrogenous lipid from horse liver. The structure of the lipid is thought to be analogous to that of lecithin, except that the base is replaced by a molecule of inositol. The structure of the polyglycerol phospholipid of horse liver (McKibbin & Taylor, 1952) has not yet been ascertained with certainty, although it may exist as a polymer and may be related to the phosphatidic acid cardiolipin, isolated by Pangborn (1947) from ox-heart muscle.

The author is indebted to Sir Rudolph Peters, F.R.S., for advice and encouragement, Dr D. N. Rhodes for much advice on column chromatography of phospholipids, Mr R. W. White for his assistance with the mass surface culture of *P. notatum* and microbiological assay of inositol, and Miss Norma Hemington for much painstaking analysis and skilled technical assistance.

SUMMARY

1. Extracts of *Penicillium notatum* and the partially-purified phospholipase *B* it contains will attack lysolecithin, but are without action on pure lecithin.

2. Such extracts attack the lecithin and phosphatidylethanolamine contained in an emulsion of crude liver lipids suspended in a buffer at pH 4, producing free fatty acids and glycerylphosphorylcholine or glycerylphosphorylethanolamine.

3. Two active lipids have been isolated from liver which cause *P. notatum* extracts to break down intact lecithin when added in concentrations as low as 30 μM.

4. One of these lipids has been identified as monophosphoinositide; the other is most probably a polyglycerol phospholipid.

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The Reported Occurrence of Sphingosylphosphorylcholine in Animal Tissues

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Little is known about the intermediary metabolism of sphingomyelin. Rossi (1935) and Goebel & Seckfort (1948) reported an uncharacterized sphingomyelinase in extracts of spleen, brain and liver, and Thannhauser & Reichel (1940) and Fujino (1952) have produced some evidence that this acts by splitting the bond between lignocerylsphingosine and phosphorylcholine. Booth (1935) and Booth & Milroy (1935) described the isolation of a water-soluble choline precursor from various animal tissues, and identified this as the choline ester of sphingosinephosphoric acid. King & Small (1939) provisionally identified a similar substance from pig pancreas as the same compound. Aloisi (1947) also isolated the substance from pancreas and studied the rate at which it was oxidized by various tissues.

It was considered by the author that if sphingosylphosphorylcholine occurred in tissues some insight into the metabolism of sphingomyelin might be obtained by studying the relationship between the phosphodiester and the phospholipid after the injection of isotopes. Analogous studies have indicated the metabolic relationship between glycerylphosphorylcholine and lecithin and also between glycerylphosphorylethanolamine and phosphatidylethanolamine in the liver of the rat (Dawson, 1955*a*). However, from fractions obtained by methods equivalent to those described by Booth (1935) and King & Small (1939) no evidence could be obtained for the presence of sphingosylphosphorylcholine, nor could the compound be detected chromatographically in various tissues of the rat or sheep. These negative results are reported because of the frequency with which the isolation of the phosphodiester is quoted in the literature, e.g. Celmer & Carter (1952), Artom (1952), Rouser, Berry, Marinetti & Stotz (1953).

Attempts were made to prepare sphingosylphosphorylcholine both by the acid and alkaline methanolysis of sphingomyelin. Whereas the results confirm the findings of Rennkamp (1949) that sphingosylphosphorylcholine is a minor component of the acid hydrolysate of sphingomyelin, none of the diester could be obtained from alkaline hydrolysates where its presence has been reported by Rouser *et al.* (1953).

EXPERIMENTAL

Materials. The sphingomyelin used was generously provided by Dr J. Olley and had been prepared from ox spinal cord (Found: N, 3·45; P, 3·8%; N:P molar ratio 2·01:1. Calc. for lignocerylsphingosylphosphorylcholine: N, 3·4; P, 3·7%; N:P molar ratio 2:1). No carbohydrate could be detected by the Molisch test. A small sample of sphingomyelin prepared by Dr D. N. Rhodes on a silicic acid column and having an N:P ratio of 2·08:1 gave the same hydrolysis products as the main sample. Phosphorylcholine and glycerylphosphorylcholine were prepared as described by Dawson (1955*b*, 1956). A solution containing sphingosylphosphorylcholine was prepared by hydrolysis of sphingomyelin (20 mg.) with 2 ml. of methanolic 1·4 N-HCl for 2 hr. in a sealed ampoule at 100°. The

hydrolysate was evaporated to dryness *in vacuo* and the residue shaken with 4 ml. of water plus 10 ml. of ether. After centrifuging, the ethereal supernatant was withdrawn, and the aqueous layer was shaken with 5 ml. of $CHCl_3$. The emulsion was centrifuged for 15 min. at 10 000 *g*, the aqueous layer was withdrawn, concentrated *in vacuo* and applied as a strip on a paper chromatogram which was developed in butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase). The sphingosylphosphorylcholine was located by spraying marker strips first with ninhydrin (0·25% in acetone) and then with a reagent for detecting phosphorus: it had an R_P of approx. 0·62. The strip containing the compound was cut out and chromatographically eluted with methanol (5 ml.). This solution was used to provide a reference marker.

Estimations. Phosphorus was determined by the procedure of Fiske & Subbarow (1925), after organic P had been converted into inorganic P by oxidation with perchloric acid (King, 1951). Nitrogen was estimated after digestion with the reagent of Hiller, Plazin & Van Slyke (1948) and distillation of NH_3 in a Pregl apparatus; occasionally digestion was carried out with the reagent of Borsook & Dubnoff (1939), followed by estimation of NH_3 in a Conway microdiffusion unit. Choline was estimated by the procedure of Appleton, La Du, Levy, Steele & Brodie (1953) and glycerylphosphorylcholine after acid hydrolysis of the labile choline in N-HCl at 100° for 20 min. Sphingosine was determined by the method of McKibbin & Taylor (1949).

Chromatography. Washing of the paper before chromatography, the method of chromatography, and the detection of phosphorus and ninhydrin-reacting spots have been described (Dawson, 1955*a*). Choline was detected on chromatograms, occasionally by the method of Chargaff, Levine & Green (1948) or more usually by the iodine-vapour procedure of Brante (1949). With the latter method it was found essential to dry thoroughly any acid solvent from the paper before applying the test, else low concentrations of choline (3–5 μg.) were not detected. Solvents used for the chromatography of free choline were those of Whittaker & Wijesundera (1952) and also butanol–acetic acid–water (4:1:5, by vol.; upper phase). For the separation of sphingosylphosphorylcholine from other methanol- and water-soluble phosphate esters which occur in tissues it was necessary to employ two-dimensional paper chromatography. Phenol saturated with water or 0·1% aq. NH_3 soln. was used in the first run; in this solvent sphingosylphosphorylcholine, glycerylphosphorylcholine and phosphorylcholine (R_P values approx. 0·9) separate from other phosphate esters which are present. The second solvent used was either butanol–acetic acid–water or ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.). In the acid solvent sphingosylphosphorylcholine had R_P approx. 0·6 whereas glycerylphosphorylcholine and phosphorylcholine both ran to R_P 0·11. In the alkaline solvent both phosphorylcholine and sphingosylphosphorylcholine had R_P 0·20 and glycerylphosphorylcholine R_P 0·45.

For the chromatographic detection of water- and methanol-soluble phosphate esters the tissue (0·5 g.) was homogenized in a blender with 25 ml. of methanol and centrifuged. The methanolic extract was evaporated to dryness *in vacuo* and the residue was shaken with 5 ml. of water and 10 ml. of ether. The mixture was centrifuged, the ethereal layer was withdrawn and the aqueous layer was shaken with 5 ml. of $CHCl_3$. After centrifuging, the aqueous layer was neutralized to pH 7, then concentrated to low volume and applied to the paper.

RESULTS

Isolation and examination of the fraction described by Booth (1935)

This fraction, described by Booth (1935) as the choline ester of sphingosine phosphoric acid, was isolated from sheep kidney and brain by essentially the same procedure. The products obtained were snow-white light solids which were readily soluble in both ethanol and water. The product from brain had an N:P molar ratio of 1·6:1 and that from kidney 1·3:1. On two-dimensional paper chromatography of the fractions in phenol–aq. NH_3 soln. and ethanol–aq. NH_3 soln., no ninhydrin-reacting and phosphorus-containing spot appeared in the sphingosylphosphorylcholine position. Phosphorus-containing spots appeared corresponding to glycerylphosphorylcholine and phosphorylcholine. There were also a number of ninhydrin-reacting spots, not containing phosphorus, corresponding with various amino acids. Again on two-dimensional paper chromatography of the fraction with phenol–aq. NH_3 soln. and butanol–acetic acid–water, no sphingosylphosphorylcholine spot was visible. Neither product contained sphingosine, when this base was estimated after the fractions had been hydrolysed for 16 hr. at 100° in 5N-HCl. Acid-labile choline determinations showed that 42% of the phosphorus in the product from kidney was present as glycerylphosphorylcholine, whereas the corresponding figure for the brain fraction was 28%. Both products contained traces of glycerylphosphorylethanolamine, revealed by paper chromatography.

Examination of the pig-pancreas fraction described by King & Small (1939)

This fraction was isolated from fresh pig pancreas as a syrupy liquid. On examination by two-dimensional paper chromatography, no sphingosylphosphorylcholine could be detected, but the major part of the phosphorus was present as a mixture of phosphorylcholine and glycerylphosphorylcholine; the concentration of the phosphodiester was 2–3 times higher than that of the monophosphoester. There were also numerous ninhydrin-reacting spots which did not contain phosphorus and which were probably amino acids.

Examination of tissues for the presence of sphingosylphosphorylcholine

The water- and methanol-soluble phosphate esters from rat kidney, brain, spleen and liver, sheep brain and pancreas, and pig pancreas were

examined for sphingosylphosphorylcholine by two-dimensional paper chromatography. In no instance was a ninhydrin-reacting phosphorus-containing spot located in the position of the diester, but this does not eliminate the possibility that very small amounts of diester may be present in these tissues.

Breakdown of sphingomyelin with methanolic acid and alkali

Rennkamp (1949) reported the occurrence of two phosphorus-containing acid-hydrolysis products of sphingomyelin, which when isolated as Reineckate salts gave analyses corresponding to phosphorylcholine and sphingosylphosphorylcholine, the diester being present as only a very minor component. The present findings agree with the results of Rennkamp on these points. On hydrolysis of sphingomyelin in methanolic 1·4N-HCl, either under reflux or in a sealed tube at 100°, two water-soluble phosphorus-containing hydrolysis products could be separated by paper chromatography with butanol–acetic acid–water. The predominant phosphorus-containing spot (R_F 0·11) was not ninhydrin-reacting and was identified as phosphorylcholine. The other phosphorus-containing spot (R_F 0·58–0·64) was strongly ninhydrin-reacting and reacted with the choline-detecting reagents of Chargaff *et al.* (1948), but as pointed out by Rouser *et al.* (1953) this spray reacts with sphingosine as well as choline. However, when the compound was eluted and hydrolysed for 18 hr. in 5N-HCl at 100°, choline was detected chromatographically among the hydrolysis products as well as inorganic phosphate and a ninhydrin-reacting substance which is presumably sphingosine. The choline and phosphorus were in equal molar ratios and the N:P ratio was approx. 2:1.

This ninhydrin-reacting phosphate ester occurred in acid hydrolysates in such low concentrations that it was possible to obtain it in solution only, but the present findings leave little doubt that it is sphingosylphosphorylcholine, thus confirming the finding of Rennkamp (1949).

Attempts were made to prepare sphingosylphosphorylcholine by hydrolysis of sphingomyelin with KOH in a sealed tube at 100° according to the directions of Rouser *et al.* (1953). The methanolic solution was freed from alkali either by passage through a column of Amberlite IRC 50 anion-exchange resin which was equilibriated with methanol, or by neutralizing the solution with methanolic HCl and removing the precipitated KCl after cooling. The methanolic solution was shown by paper chromatography to contain, as well as unchanged sphingomyelin, a ninhydrin-positive and phosphorus-containing spot similar to that of sphingosylphosphorylcholine, but in the ethanol–aq. NH_3 soln. solvent it remained at the origin, whereas the diester isolated from the acid hydrolysate had an R_F of about 0·2. The compound was insoluble both in $CHCl_3$ and water and, when shaken with these two solvents and centrifuged, it formed a white pellicle between the two layers of solvent. On dissolving this in $CHCl_3$–methanol (1:1, v/v) it could be reprecipitated with 3 vol. of acetone. On drying, the product became resinous and brown, and all attempts to crystallize it were unsuccessful. Two successive preparations had P:N molar ratios of 1:0·97 and 1:0·96, and on total hydrolysis in 5N-HCl at 100° such that all the phosphorus was converted into the inorganic form, no free choline could be detected in the hydrolysate. Although the identity of the compound was not ascertained it was clearly not sphingosylphosphorylcholine. The phosphorus content of the preparations (4·6, 5·0%) was too low for that of sphingosine phosphate. No evidence was obtained that the methanolic-KOH hydrolysate of sphingomyelin contained more than traces of water-soluble phosphorus-containing hydrolysis products, and sphingosylphosphorylcholine could not be detected.

DISCUSSION

The finding that glycerylphosphorylcholine is present as the main component in the fraction described by other workers as sphingosylphosphorylcholine is perhaps not surprising, as this former compound has comparatively recently been described as a constituent of animal tissues (King & Aloisi, 1945; Schmidt, Hershman & Thannhauser, 1945; Schmidt, Greenbaum, Fallot, Walker & Thannhauser, 1955). Moreover, free phosphorylcholine is known to be widely distributed in appreciable concentration in various tissues (Dawson, 1956). Both these phosphate esters are relatively soluble in water and ethanol and are not precipitated from aqueous solutions by common heavy-metal ions such as Ba^{2+}, Hg^{2+} or Pb^{2+}. Moreover, the present investigation has shown them to be precipitated from ethanolic solution with mercuric chloride, one of the purification steps used in the Booth (1935) procedure. King & Aloisi (1945) found that glycerylphosphorylcholine was isolated from dried ox pancreas by a method similar to that which yielded 'sphingosylphosphorylcholine' from fresh pig pancreas, and Schmidt *et al.* (1945) suggested that glycerylphosphorylcholine, together with a nitrogenous impurity, might account for the material in the fraction isolated by King & Small (1939).

Neither Booth (1935) nor King & Small (1939) positively identified sphingosine in their samples, but at the time no method was available for the estimation or detection of sphingosine, nor was

paper chromatography available. The suggestion that sphingosine was present was made by Booth (1935) because of the total N and amino-N analysis of the isolated samples, which gave an N:P molar ratio of 2:1; in the present work a puzzling factor has been that the N:P molar ratios of the equivalently isolated fractions were lower than 2:1. Chromatographic results of the present paper suggest that sphingosylphosphorylcholine is not present in any of the animal tissues examined in any appreciable concentration, but its presence as a very minor constituent cannot be excluded.

The failure to confirm the chromatographic findings of Rouser *et al.* (1953) of the presence of sphingosylphosphorylcholine in alkaline hydrolysates of sphingomyelin is somewhat puzzling. The main if not the sole hydrolysis product, although ninhydrin-reacting and phosphorus-containing, contains no choline and possesses equimolar proportions of nitrogen and phosphorus. No chromatographic spot equivalent to sphingosylphosphorylcholine was detectable in the hydrolysate. It is suggested that a complete chemical investigation of the alkaline hydrolysis of sphingomyelin is necessary before the confusion in this subject can be clarified.

SUMMARY

1. Fractions of animal tissues which were reported by other workers to contain the choline ester of sphingosine phosphoric acid were isolated by equivalent methods and analysed.

2. The fractions contained glycerylphosphorylcholine, phosphorylcholine and amino acids in varying proportions but no sphingosylphosphorylcholine.

3. Sphingosylphosphorylcholine was not detected chromatographically in the water- and methanol-soluble phosphate esters extracted from tissues.

4. Methanolic hydrochloric acid treatment of sphingomyelin yielded phosphorylcholine and a small amount of sphingosylphosphorylcholine. Methanolic-potassium hydroxide hydrolysis gave an unidentified water- and chloroform-insoluble compound with phosphorus and nitrogen in equimolar proportions: no sphingosylphosphorylcholine was detected in the alkaline hydrolysate.

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Studies of the Metabolism *in vivo* of Cortisol by the Rat Liver

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The metabolism of cortisol (Caspi & Hechter, 1956) and cortisone (Caspi, Levy & Hechter, 1953; Caspi & Hechter, 1954; Miller & Axelrod, 1954) has been studied in the rat liver with the isolated perfused organ. In these experiments the hormones were dissolved in either homologous or heterologous blood, which was then perfused through the liver and the effluent extracted for the steroid metabolites. Since it is possible that the results obtained by this technique might be different from those in which cortisol metabolites are isolated from liver *in situ*, the metabolism of cortisol by the liver was studied after its parenteral administration in the rat.

EXPERIMENTAL

Nomenclature

Cortisol, cortisone, corticosterone, β-cortol, β-cortolone, α-cortolone and tetrahydrocortisol are used as trivial names for 11β:17α:21-trihydroxypregn-4-ene-3:20-dione, 17α:21-dihydroxypregn-4-ene-3:11:20-trione, 11β:21-dihydroxypregn-4-ene-3:20-dione, 3α:11β:17α:20β:21-pentahydroxypregnane, 3α:17α:20β:21-tetrahydroxypregnan-11-one, 3α:17α:20α:21-tetrahydroxypregnan-11-one, and 3α:11β:17α:21-tetrahydroxypregnan-20-one respectively throughout this paper.

Materials

Radioactive cortisol. [4-^{14}C]Cortisol (specific activity 1·467 mc/m-mole), supplied by the National Institutes of Health, U.S. Public Health Service, was purified by paper chromatography by development for 6 days in the benzene–formamide system. The area corresponding to cortisol was cut out and eluted.

Non-radioactive cortisol. Sterile solutions of 'hydrocortisone (free alcohol) intravenous' (Boots Pure Drug Co. Ltd.) in ampoules which contained 100 mg. in 20 ml. of 50% (v/v) ethanol, were diluted to 50 ml. with aq. 0·9% NaCl soln.

Reagents and solvents. Silica gel was used untreated. Ethyl acetate was washed once with one-quarter of its volume of aq. 10% Na_2CO_3 soln., twice with one-quarter of its volume of water and dried over anhydrous Na_2SO_4. Solvents for paper chromatography were used as supplied.

Animals. Male albino rats (Glaxo strain) weighing 200–350 g. were used.

Chromatography

Solvent systems. All chromatograms were developed in a descending manner on Whatman no. 1 filter paper in all-glass tanks at 23±2°. Extracts were applied 4¾ in. from the end of the 22½ in.-long paper, and after development the chromatograms were dried in an oven at less than 40°. The solvent systems used, and abbreviations used for them in the text, are listed: Benzene–formamide (Burton, Zaffaroni & Keutmann, 1951); B-5: benzene–methanol–water (2:1:1, by vol.) (Bush, 1952); B-5EtAc: benzene–methanol–water–ethyl acetate (1:1:1:0·1, by vol.) (Hübener, Fukushima & Gallagher, 1956); E_2B: 2:2:4-trimethylpentane–*tert.*-butyl alcohol–water (10:5:9, by vol.) (Eberlein & Bongiovanni, 1955); LB21/80: light petroleum (b.p. 80–100°)–benzene–methanol–water (10:5:12:3, by vol.) (I. E. Bush, personal communication); L/96: light petroleum–methanol–water (100:96:4, by vol.) (I. E. Bush, personal communication); butanol–water: aq. 86% (v/v) *m*-butanol (Markham & Smith, 1949); butanol–water–ammonia: aq. 86% (v/v) *n*-butanol with 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·880) added to solvent in the bottom of the tank (Markham & Smith, 1949).

Detection of steroids on paper chromatograms. Unsaturated steroids were examined briefly under a lamp (the Chromatolite, Hanovia Lamps, Slough) emitting most of its energy at 253·7 mμ. Δ^4-3-Ketones were detected by the soda fluorescent reaction described by Bush (1952). Steroids possessing an α-ketol side chain were detected with triphenyltetrazolium chloride (Burton *et al.* 1951). 17α:20:21-Triol compounds were oxidized to 17-oxo compounds by dipping a paper strip once in a 4:1 (v/v) solution of absolute ethanol–4% aq. sodium metaperiodate and hanging it horizontally for 40 min. at room temperature. In order to detect the resulting 17-oxo steroid it was dipped once in a 2:1 (v/v) mixture of ethanolic 2% *m*-dinitrobenzene soln. and ethanolic 4N-KOH soln. (modified Zimmermann reagent), dried briefly in a draught and then heated in an oven for 2–3 min. at approx. 50°.

Elution of steroids from paper chromatograms. The area of the paper to be eluted was cut into pieces approx. 2 cm.^{2} and these were placed into glass-stoppered test tubes. Sufficient absolute ethanol was added to cover them and the tubes were shaken for 1 hr. The ethanol was removed with a Pasteur pipette, filtered with suction through a sintered-glass funnel into a 10 ml. centrifuge tube and evaporated to dryness with a stream of air at 45–50°. This process was repeated twice.

Spectrophotometry

Absorption spectra were determined in an SP. 500 spectrophotometer (Unicam Instruments Ltd.) with silica cells with a 0·2 cm. light path. The absorption spectra of

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steroids in conc. H_2SO_4 were determined by the method of Zaffaroni (1950). Infrared spectra were determined in a model 21 double-beam infrared spectrophotometer (Perkin–Elmer Corp., Norwalk, Conn.) with a KCl microplate with 150–200 μg. of the free steroid alcohol.

Oxidation and acetylation

Sodium bismuthate oxidation was carried out by the method of Brooks & Norymberski (1953). Chromic acid oxidation was carried out in the following manner: to the dry steroid, 0·3 ml. of acetic acid and 0·2 ml. of 2% (w/v) aq. chromium trioxide soln. were added, and the solution was left at room temperature for approx. 21 hr. A small volume (2 ml.) of water was added to the solution, which then was extracted four times with 2 ml. of ethyl acetate each time, and the pooled extract was evaporated to dryness with a stream of air at 45–50°. Acetates were formed by adding three drops of acetic anhydride and five drops of absolute pyridine to the dry steroid and letting it stand at room temperature for 15–18 hr. After addition of a small amount of methanol, the acetates were evaporated to dryness with a stream of air at 45–50°.

Experiments with [4-^{14}C]*cortisol*

Approx. 0·8 μc of [4-^{14}C]cortisol was dissolved in 0·9 ml. of a 20% (v/v) ethanolic 0·9% NaCl soln. and injected via the right external jugular vein into a 200 g. rat which had been anaesthetized by an intraperitoneal injection of sodium pentobarbitone (5 mg./100 g. body wt.). Three minutes after the end of the injection, the rat was exsanguinated by withdrawing blood from the inferior vena cava into a heparinized syringe. A second rat was exsanguinated 10 min. after the end of the injection. Immediately thereafter the animal was perfused with 60–70 ml. of 0·9% NaCl soln., first via the inferior vena cava at a point just below the entrance of the renal veins and then via the abdominal aorta. The right external jugular vein was cut after the start of the perfusion. When the fluid coming out of the jugular vein was colourless and the liver was pale pink the perfusion was stopped, and the liver removed and stored at −20° until it was extracted.

The liver was thawed, put into a 10 ml. glass cup which was surrounded by cracked ice, and homogenized (MSE homogenizer) with 2 ml. of 0·9% NaCl soln. Immediately after the liver was homogenized it was extracted four times with 25 ml. of ethyl acetate each time, and the pooled extract distilled *in vacuo* at 40–45° to a volume of approx. 2 ml. The concentrated extract was dissolved in 25 ml. of light petroleum (b.p. 40–60°)–ethyl acetate (1:1, v/v) and chromatographed on 2 g. of silica gel, as described by Bush & Sandberg (1953). The eluate from the silica-gel column was evaporated to dryness with a stream of air at 45–50°. To the dried eluate was added 0·1 ml. of absolute ethanol, which then was applied to the paper together with three 0·05 ml. washings. The paper was developed for 6 days in the benzene–formamide system and the overflow after being evaporated to dryness and redissolved in ethanol was chromatographed for 3 hr. in the same system. Radioautographs were obtained by placing the paper chromatograms in contact with Blue-brand X-ray film (Kodak Ltd.) for 14 days and developing the films by standard photographic procedures.

Experiments with non-labelled cortisol

The object of the experiments with ^{14}C-labelled cortisol was to obtain some information on (1) the number of cortisol metabolites which were present in liver after parenteral administration of the hormone, (2) the relative polarity of these metabolites, so as to get some idea of their structure, and (3) the relative amounts of these metabolites. Since most of the radioactivity was concentrated in two or more very polar compounds, an attempt was made to identify them by injecting rats with large amounts of non-radioactive cortisol. The following experiments were designed with this purpose in mind.

Rats anaesthetized with sodium pentobarbitone were injected with 8 mg. of cortisol in 20% ethanolic 0·9% NaCl soln. via the right external jugular vein, at a rate of approx. 1 mg./min. Twenty minutes after the start of the injection, the rats were exsanguinated and perfused as described in the previous section. In the first of these experiments ethyl acetate extracts of the livers were chromatographed on silica gel, as described above, to remove large amounts of fatty non-steroidal material. When it was found that washing the extracts with hexane was quicker and achieved similar results to those obtained by partition on silica gel, the following procedure was adopted.

The combined ethyl acetate extracts from the livers of six to eight rats were concentrated to a volume of 0·5–1 ml. by distillation *in vacuo* at 40–45°. The extract then was dissolved in 50 ml. of aq. 70% methanol soln., and washed six times with 100 ml. of hexane. Methanol was removed by distillation *in vacuo* and the resulting solution extracted six times with 15 ml. of ethyl acetate. The combined extract was concentrated as before and transferred to a 10 ml. centrifuge tube with several washings of methanol. After the solution was evaporated to dryness with a stream of air at 45–50°, it was ready to be chromatographed on paper.

RESULTS

Experiments with 4-^{14}C-*labelled cortisol*

Radioautographs from the chromatograms developed for 6 days showed that most of the radioactivity was concentrated just in front of the origin in two poorly resolved spots whose R relative to cortisol was 0·07 and 0·21. Two very weak radioactive areas were also seen whose R relative to cortisol was 0·37 and 0·60. With the exception of a radioactive area with the same mobility as a standard spot of cortisol, the only other radioactive spot seen was a barely visible one with an R relative to cortisol of 1·8.

No radioactive areas could be detected in the radioautograph from the benzene–formamide chromatogram developed for 3 hr.

Experiments with non-labelled cortisol

In two radioactive-hormone experiments, examination of the benzene–formamide chromatograms developed for 6 days with a lamp emitting most of its energy at 253·7 mμ showed a red-fluorescing

area just in front of the origin, which corresponded exactly to the two poorly resolved strongly radioactive spots seen on the radioautograms. When this area was rechromatographed for 18 hr. in the B-5 system, the radioactive and fluorescent areas still were superimposed. Chromatography in the B-5 system for 72 hr. resolved the u.v. light-fluorescing area into two spots, one of which (compound NP) had moved 5·75 cm. from the origin, whereas the other (compound P), which appeared to be present in a much smaller quantity as judged by the intensity of the fluorescence, was located 2·5 cm. from the origin.

The absorption spectrum of compound NP in absolute ethanol showed a maximum at 262 mμ with a shoulder between 256 and 258 mμ and a minimum between 245 and 246 mμ, whereas the absorption spectrum of compound P in absolute ethanol revealed a maximum between 257 and 258 mμ and a minimum at 238 mμ. When the solution of compound NP was evaporated to dryness with a stream of air and the compound dissolved in 0·1 N-HCl, the absorption maximum and minimum had shifted to 260 and 238mμ respectively and the shoulder had disappeared. The compound could not be extracted into ethyl acetate from the 0·1 N-HCl solution. The absorption spectrum of compound NP in N-NaOH was identical with that in absolute ethanol, and the compound could be extracted into ethyl acetate from the alkaline solution.

Since these experiments indicated that compound NP might be a nucleotide, it was chromatographed on paper for 22 hr. in butanol–water–aq. NH_3 soln. and its R_F found to be 0·67 (R_F values of adenine, cortisol, corticosterone and 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one were 0·37, 0·91, 0·94 and 0·87 respectively). After hydrolysis in N-HCl at 110° for 2 hr., the R_F of compound NP in the same system shifted to 0·22 (R_F values of adenine, xanthine and hypoxanthine were 0·37, 0·05 and 0·13 respectively), but its absorption spectrum in absolute ethanol was unchanged.

In order to determine whether the radioactive material could be separated from the u.v. light-fluorescing material by chromatography in a solvent system not generally used for adrenocortical steroids, the very polar area present in the benzene–formamide chromatogram developed for 6 days was eluted and rechromatographed for 16 hr. in butanol–water. A radioautogram showed a single radioactive spot with R_F 0·91, and examination of the chromatogram with a u.v. light lamp (253·7 mμ) revealed a single fluorescing spot with R_F 0·67. A compound was also found in the livers of rats not given cortisol which had properties identical with those of the compound isolated from the livers of rats injected with cortisol. Since this compound appeared to be a normal constituent in rat liver and not to be a metabolite of cortisol, no further attempt was made to identify it.

Paper-chromatographic separation of cortisol metabolites

The complete separation of the strongly u.v. light-fluorescing compound from the cortisol metabolites appeared to be possible by chromatographing the liver extracts in butanol–water. However, it was found that when the extracts from six to eight livers were chromatographed on a single sheet of paper in this system, such a large quantity of this u.v. light-fluorescing compound was extracted that even though the steroids moved virtually with the solvent front, some of this compound overlapped the area on the chromatogram where the steroids were located. Therefore after the very polar metabolites of cortisol were separated from each other, the u.v. light-fluorescent compound which was present as a contaminant in each of these polar steroids was separated from the latter by chromatography in the E_2B system. The sequence of paper chromatography which was finally used was the following.

The hexane-washed liver extracts were chromatographed in the B-5 system for 24 hr. and the chromatogram was divided into four zones: zone I (from the origin up to and including the area corresponding to 11β:17α:20β:21-tetrahydroxypregn-4-en-zone; zone II (the area just in front of 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one up to but not including the cortisol area); zone III (the area corresponding to cortisol); zone IV (the area in front of cortisol).

Zone I was eluted and rechromatographed for 48 hr. in the B-5EtAc system. In this system, 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one, β-cortolone, 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane and β-cortol (in order of increasing polarity in B-5EtAc) were completely separated from each other. Each of the metabolites found in zone I (compounds 1–4, see below) was eluted and rechromatographed in the E_2B system for 72 hr.

Metabolites in zone I

Identification of compound 1 *as β-cortol* (3α:11β:17α:20β:21-*pentahydroxypregnane*). When this substance was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent, it gave a positive test indicating the possible presence of a 17α:20:21-triol-type of side chain. The compound had the same mobility as an authentic sample of β-cortol when it was chromatographed for 72 hr. in both the B-5EtAc and the E_2B systems, and a mixed chromatogram developed for 72 hr. in the B-5EtAc system gave a single spot. Oxidation with sodium bismuthate

resulted in a compound which had the same mobility on paper as an authentic sample of 3α:11β-dihydroxy-5β-androstan-17-one when chromatographed for 24 hr. in the LB 21/80 system. When the compound oxidized with sodium bismuthate was acetylated and chromatographed for 13 hr. in the L/96 system, its mobility was the same as 3α-acetoxy-11β-hydroxy-5β-androstan-17-one. When the acetylated compound oxidized with sodium bismuthate was further oxidized with chromic acid, a compound was formed which when chromatographed for 7·5 hr. in the L/96 system had the same mobility as an authentic sample of 3α-acetoxy-5β-androstane-11:17-dione. The sulphuric acid and infrared spectra of compound 1 were identical with those of an authentic sample of β-cortol except for a maximum at 1675 cm.$^{-1}$ in the infrared-absorption spectrum of compound 1, the presence of which is difficult to explain.

Identification of compound 2 *as* 3β:11β:17α:20β:21-*pentahydroxy-5α-pregnane.* The chromatographic behaviour on paper of this compound in the B-5EtAc and E_2B systems was identical with that of an authentic sample of 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane. After oxidation with sodium bismuthate its mobility in the LB21/80 system was the same as that of 3β:11β-dihydroxy-5α-androstan-17-one, and acetylation of the compound oxidized with bismuthate resulted in a substance which had the same mobility in the L/96 system as 3β-acetoxy-11β-hydroxy-5α-androstan-17-one. When the acetylated compound oxidized with sodium bismuthate was further oxidized with chromic acid, a compound was formed which when chromatographed for 7·5 hr. in the L/96 system had the same mobility as an authentic sample of 3β-acetoxy-5α-androstane-11:17-dione. In the L/96 system, although 3β-acetoxy-5α-androstane-11:17-dione has the same mobility as 3α-acetoxy-5α-androstane-11:17-dione, these two compounds run behind 3α-acetoxy-5β-androstane-11:17-dione. Similarly, 3β-acetoxy-11β-hydroxy-5α-androstan-17-one and 3α-acetoxy-11β-hydroxy-5α-androstan-17-one have the same mobility in the L/96 system but they run behind 3α-acetoxy-11β-hydroxy-5β-androstan-17-one. The sulphuric acid and infrared spectra of compound 2 were identical with those of an authentic sample of 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane.

Partial identification of compound 3 *as* 3α:17α:-20β:21-*tetrahydroxy-5α-pregnan-11-one.* This compound gave a positive test when it was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent, indicating the possible presence of a 17α:20:21-triol-type of side chain. Its mobility in the B-5EtAc system was the same as that of 3α:17α:20β:21-tetrahydroxypregnan-11-one and a mixed chromatogram with an authentic sample of the latter compound gave a single spot in the B-5EtAc system. The absoption spectrum in sulphuric acid of compound 3 was identical with that of 3α:17α:20β:21-tetrahydroxypregnan-11-one. However, when compound 3 was oxidized with sodium bismuthate and chromatographed on paper for 24 hr. in the LB21/80 system, its mobility was the same as that of 3α-hydroxy-5α-androstane-11:17-dione, and acetylation of the compound oxidized with bismuthate resulted in a substance with the same mobility as 3α-acetoxy-5α-androstane-11:17-dione in the L/96 system. Unfortunately an authentic sample of 3α:17α:20β:21-tetrahydroxy-5α-pregnan-11-one could not be obtained, and the amount of compound 3 isolated from liver was not sufficient for an infrared absorption spectrum.

Partial identification of compound 4 *as* 11β:17α:20β:21-*tetrahydroxypregn-4-en-3-one.* On paper this compound gave a positive soda-fluorescence reaction (Bush, 1952) indicating the presence of the Δ^4-3-ketone grouping, and it gave a positive test when it was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent. It exhibited a u.v. light maximum of 242–243 mμ when its absorption spectrum was determined in absolute ethanol. When compound 4 was chromatographed in the B-5EtAc system for 48 hr. and in the E_2B system for 72 hr., its mobility was the same as that of an authentic sample of 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one, and mixed chromatograms in both systems gave single spots. The absorption spectrum in sulphuric acid of compound 4 was similar to that of 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one. An infrared-absorption spectrum of compound 4 was not made because not enough of the compound could be isolated from liver.

Metabolites in zone II

Two compounds (compounds 5 and 6), which reacted with triphenyltetrazolium chloride to give a red colour and which gave a negative soda-fluorescence reaction, were found in the area corresponding to tetrahydrocortisol. One of these, compound 5, had a mobility which was slightly less than that of tetrahydrocortisol when chromatographed for 24 hr. in the B-5 system and also when chromatographed for the same length of time in the B-5EtAc system. When compound 5 was oxidized with sodium bismuthate and chromatographed for 24 hr. in the LB 21/80 system, its mobility was slightly greater than that of 3β:11β-dihydroxy-5α-androstan-17-one and it ran behind 3α:11β-dihydroxy-5β-androstan-17-one. Acetylation of the compound oxidized with sodium bismuthate resulted in a substance which, when developed for 4 hr. in

the L/96 system, ran just behind 3α-acetoxy-11β-hydroxy-5α-androstan-17-one. The sulphuric acid spectrum of compound 5 showed maxima at 338–342, 412–413, 505–508 mμ and minima at 264–266, 381–382 and 475 mμ.

Compound 6 had a mobility which was slightly greater than that of tetrahydrocortisol when chromatographed for 24 hr. in the B-5 system. After oxidation with sodium bismuthate, compound 6 ran just in front of 3α:11β-dihydroxy-5β-androstan-17-one in the LB 21/80 system and acetylation of the compound oxidized with sodium bismuthate resulted in a substance with a mobility in the L/96 system which was slightly greater than that of 3α-acetoxy-11β-hydroxy-5β-androstan-17-one.

Metabolites in zone III

Partial identification of compound 7 as cortisol (11β:17α:21-*trihydroxypregn*-4-*ene*-3:20-*dione*). On the paper chromatogram this compound gave a positive soda-fluorescence reaction, and in both the B-5 and the benzene–formamide systems its mobility was the same as that of an authentic sample of cortisol. It exhibited a u.v. light maximum at 240 mμ when its absorption spectrum was determined in absolute ethanol.

Metabolites in zone IV

Traces of a compound which gave a positive soda-fluorescence reaction, and which had the same mobility as cortisone in the B-5 system, were found in this zone.

Quantities of metabolites identified

The relative quantities of steroid metabolites were estimated by visual comparison on paper with known amounts of reference compounds, periodate oxidation followed by the Zimmermann reaction being used for the 17α:20:21-triol type of compounds and the triphenyltetrazolium chloride reaction for the tetrahydro type of compounds. Visual comparison with β-cortolone was used for estimating the quantity of the compound partially identified as 3α:17α:20β:21-tetrahydroxy-5α-pregnan-11-one and comparison with tetrahydrocortisol was used for estimating the two tetrahydrocortisol-like metabolites. Metabolites with the α–β unsaturated ketone group were estimated after determining in absolute ethanol the optical density at the u.v. light maximum by the use of the known molar-extinction coefficients of the compounds.

It was estimated that from each rat liver were isolated 150–200 μg. of β-cortol, 100–150 μg. of 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane, 5–8 μg. of 3α:17α:20β:21-tetrahydroxy-5α-pregnan-11-one, and 1–5 μg. each of 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one, cortisol and the two tetrahydrocortisol-like compounds.

DISCUSSION

It should be emphasized that the cortisol metabolites from liver which have been detected and identified in this study do not necessarily give a complete picture of the metabolic fate of this hormone, conjugation excluded. Caspi & Hechter (1956), who perfused [4-^{14}C]cortisol through isolated rat livers, were able to extract only 55% of the initially added radioactivity from the effluent, and Cope & Sewell (1956), studying the metabolism of [4-^{14}C]cortisol in the mouse, found that up to 60% of the radioactivity in the whole animal had been converted into products which could not be extracted into chloroform even after acid hydrolysis.

The data obtained in the present study confirm, at least in part, the perfusion experiments of Caspi & Hechter (1956), who showed that rat liver could reduce the Δ^4-3-ketone group of cortisol to 3β-hydroxylated metabolites, and also reduce the carbonyl group at C-20 to the 20β-hydroxy grouping. However, whereas the three major transformation products in Caspi & Hechter's study were 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane, 3β:11β:17α:21-tetrahydroxy-5α-pregnan-20-one and 11β:17α:20β:21-tetrahydroxypregnan-4-en-3-one (39, 19 and 15% respectively of the metabolites isolated) in the experiments described in this paper, it is estimated that 80–90% of the cortisol metabolites were accounted for by 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane and β-cortol. Only very small amounts of metabolites were found with the Δ^4-3-ketone grouping and reduction of the C-20 carbonyl group (11β:17α:-20β:21-tetrahydroxypregn-4-en-3-one), or reduction of the Δ^4-3-ketone group but with the C-20 carbonyl group intact ('tetrahydro' group of metabolites).

Of the two principal cortisol metabolites identified in the present study *in vivo*, one was found to be a 3β-alcohol and the other a 3α-alcohol. Caspi *et al.* (1953), Caspi & Hechter (1954) and Miller & Axelrod (1954) also identified 3β- and 3α-metabolites in the effluent from rat livers perfused with cortisone. However, in their perfusion of cortisol through rat liver, Caspi & Hechter (1956) were able to isolate only metabolites with the 3β-configuration. This is difficult to understand in view of the work of Tomkins (1956), who was able to isolate and partially purify a 3α-hydroxysteroid dehydrogenase from rat liver.

In the liver-perfusion experiments of Caspi *et al.* (1953), Caspi & Hechter (1954, 1956) and Miller & Axelrod (1954) with cortisone and cortisol the

metabolites in which the Δ^4-3-ketone group had been reduced were all of the 5α-configuration. This was not found to be so in the present experiments in which metabolites with a saturated A ring had both the 5α- and 5β-configuration. The data obtained in this study accord better with the finding of Forchielli & Dorfman (1956), who reported that rat liver contains both the Δ^4-5α-hydrogenase and the Δ^4-5β-hydrogenase systems.

One further point of difference between the data obtained from liver-perfusion experiments and the data presented in this paper is that Caspi & Hechter (1956) identified two 17-oxosteroids in their effluents: 3β:11β-dihydroxyandrostan-17-one and 11β:hydroxyandrosten-4-ene-3:17-dione, whereas in the present study no 17-oxosteroids were found. If any 17-oxosteroids had been present they would have been detected as radioactive spots when the overflow from the benzene–formamide chromatogram developed for 6 days was chromatographed for 3 hr. in the same system. This discrepancy, together with the others which have been described above, possibly may be due to the differences in the experimental methods employed, one of which is essentially a technique *in vitro*, whereas the other is *in vivo*. In a recent discussion on the possible mechanisms of hormone action (Hechter, 1955) it was pointed out that many of the effects of adrenocortical hormones observed *in vivo* could not be obtained *in vitro*, and Hechter, Solomon & Caspi (1953) emphasized that liver perfusion was 'not necessarily a system for studying liver action upon corticosteroids under physiological conditions'.

Although only small amounts of what is probably 3α:17α:20β:21-tetrahydroxy-5α-pregnan-11-one were partially identified in the livers of rats injected with cortisol, it would appear that rat liver *in vivo* is capable of oxidizing, possibly only to a limited extent, the 11β-hydroxy group of cortisol to an 11-oxo group. That such a reaction undoubtedly also takes place in humans was shown by Fukushima *et al.* (1955), who isolated 3α:17α:-20α:21-tetrahydroxypregnan-11-one and its 20β-epimer from the urine of human patients after administration of adrenocorticotropic hormone and [4-^{14}C]cortisol. Gray & Lunnon (1956) also showed that in patients with Addison's disease or after adrenalectomy cortisone appears in the urine after administration of cortisol.

In this study, as well as with liver perfusion, it has been shown that two of the principal transformations occurring in the cortisone and cortisol molecules after passage through the liver are reduction of the Δ^4-3-ketone group or reduction of the C-20 carbonyl group or both. It has also been found that reduction at one or both of these positions markedly reduces the biological activity of these hormones when they are administered parenterally (Abelson, Ulrich & Long, 1955; Hechter, 1955). Therefore since cortisone and cortisol disappear from the blood (Tomizawa, Narahara, Gibbons & Williams, 1954; Ulrich & Long, 1956) and liver (Hechter, 1955) at such a rapid rate and are transformed into physiologically less active or inactive metabolites, it is difficult to understand how an adrenalectomized rat can be maintained on a single daily dose of cortisol. It has been suggested that adrenocortical hormones 'are not active at the cellular level unless they are first transformed by some other organ system into the active agent' and some evidence for this has been obtained (Hechter, 1955). It is possible that such physiologically active metabolite(s) may be found in that fraction which Caspi & Hechter (1956) and Cope & Sewell (1956) were not able to extract with organic solvents (see beginning of Discussion).

SUMMARY

1. The metabolism of cortisol in the liver of the rat was studied by intravenously administering [4-^{14}C]cortisol and non-labelled cortisol and identifying the unconjugated metabolites in the organ.

2. 3α:11β:17α:20β:21-Pentahydroxypregnane and 3β:11β:17α:20β:21-pentahydroxy-5α-pregnane were found to be the two principal metabolites occurring in the liver, and they were estimated to account for 80–90% of the metabolites found. Small amounts of cortisol, 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one, 3α:17α:20β:21-tetrahydroxy-5α-pregnan-11-one and two tetrahydrocortisol-like compounds were also detected but the quantities were too small for definite identification.

3. The results obtained in this study are compared with those obtained from liver-perfusion experiments, and the differences in the metabolites identified by the two techniques are discussed.

I am greatly indebted to Professor A. Neuberger, F.R.S., for his helpful advice and for providing the facilities of his department in which this study was carried out. I would also like to thank Dr R. K. Callow for recording and interpreting the infrared spectra and for his generous gifts of non-labelled cortisol. Dr D. K. Fukushima, Dr W. K. Klyne, Dr O. Mancera, Dr M. Tishler and Dr J. J. Schneider generously provided reference steroids, and the Endocrinology Study Section, National Institutes of Health, U.S.A., supplied the 4-^{14}C-labelled cortisol.

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Hormones and Liver Cytoplasm

5. ENZYMES CONCERNED IN NUCLEIC-ACID CATABOLISM, AS AFFECTED BY HYPOPHYSECTOMY OR ADRENALECTOMY*

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Stevens & Reid (1956) found that hypophysectomy or adrenalectomy increased the activities, assayed at pH 5, of deoxyribonuclease (DNA-ase) and ribonuclease (RNA-ase) in rat-liver cytoplasm. There were no corresponding increases in the activity of a second ribonuclease with optimum activity at about pH 8. Hypophysectomy also induced an increase in xanthine oxidase activity, whereas adrenalectomy decreased this activity (Reid, O'Neal & Lewin, 1956*a*). These effects of hypophysectomy were not altered by administration of growth hormone.

These studies have now been extended to include the related enzymes, nucleotidase, adenosine deaminase, purine nucleoside phosphorylase and uricase. To assess the bearing of changes in enzymic activity on ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) metabolism, kidney tissue has now been studied with respect to possible changes in nuclease activities, and liver tissue has been studied (as briefly reported by Reid & Stevens, 1956) with respect to the rapidity with which nucleases and other constituents change after adrenalectomy. Since the rises in liver-nuclease activities after hypophysectomy, and especially after adrenalectomy, were due in part to rises in the normally low activity of the supernatant fraction separated from the cytoplasm by differential centrifuging (Stevens & Reid, 1956), the supernatant fraction has been studied as well as the whole cytoplasmic fraction.

* Part 4: Reid & Stevens (1957).

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EXPERIMENTAL

Animals and tissue-fractionation technique

Reference should be made to Reid, O'Neal, Stevens & Burnop, 1956*b* for a full description of the general procedures. The rats were males of about 250 g. body wt. which had been given, in restricted amount, a '20% protein diet' (Elson, 1952) enriched by the addition (per 100 g. dry wt.) of 3·5 μg. of aneurin, 2·4 μg. of riboflavin, 8·2 μg. of calcium pantothenate, 3 μg. of pyridoxine hydrochloride and 588 μg. of choline chloride. The rats were fasted overnight before autopsy. The hypophysectomized rats were killed 2–3 weeks after operation, and the adrenalectomized rats (which had been maintained with saline) were, unless otherwise stated, killed 10–21 days after operation. To minimize the effect of possible day-to-day variations in environment or technique, the experimental rats were always compared with control rats (intact or sham-operated) investigated simultaneously. At least five untreated hypophysectomized rats, four hypophysectomized rats treated with growth hormone and six adrenalectomized rats were used in the study of each enzyme. Each point on Fig. 1 represents data for two to six adrenalectomized rats.

Samples of perfused liver were homogenized in 0·25 M-sucrose soln. with a plastic pestle rotating at 2000 rev./min.,

and the debris (containing nuclei) obtained by centrifuging for 10 min. at 600 *g* was rehomogenized to reduce the content of unbroken cells. A portion of the whole cytoplasmic fraction thus obtained was centrifuged as in the experiments of Reid (1956) and Reid *et al.* (1956*a*) to obtain a mitochondrial fraction (sedimented after 15 min. at 12 000 *g*), a microsomal fraction and a supernatant fraction containing the material which failed to sediment in 90 min. at 20 000 *g*. Although this gravitational force is lower than that used in some laboratories, the supernatant fraction contains little microsomal material, as judged by data for RNA synthesis (Reid & Stevens, 1957), and contains acid RNA-ase and DNA-ase in amounts (relative to the whole cytoplasmic fraction) which are respectively as low as and even lower than those reported by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), (Stevens & Reid, 1956).

The experiments with kidney were, in most instances, performed with tissue which had been stored at −30°. The tissue was homogenized once only, and a portion of the homogenate was studied without removal of the nuclei. The remainder was immediately centrifuged for 30 min. at 60 000 *g* to obtain a supernatant fraction, the sedimented material being discarded.

Determination of enzymes and other constituents

DNA was determined by alkaline digestion followed by determination of acid-insoluble phosphorus (Reid, 1956). All phosphate determinations were made according to King (1951) with ascorbic acid as reducing agent.

In the enzymic assays, which were carried out in duplicate or triplicate, proportionality was demonstrated between amount of tissue and activity. The time course of the reactions showed little departure from linearity over the time interval employed. As in the experiments of Reid *et al.* (1956*a*) and of Stevens & Reid (1956), the assays were usually performed on tissue fractions which had been frozen and stored at −30°. This procedure did not impair enzymic activity, although that of nucleoside phosphorylase was found to be impaired by repeated freezing. Nucleotidase activity was enhanced by freezing, as was the adenosine triphosphatase (ATP-ase) activity studied by Reid *et al.* (1956*a*). The nuclease assays were carried out as described by Stevens & Reid (1956), although recent experiments (Maver & Greco, 1956; J. T. Nodes & E. Reid, unpublished work) suggest that Mg^{2+} ions need not have been added, all tissue fractions being frozen and thawed eight times to ensure liberation of latent activity (de Duve *et al.* 1955); this precaution was later shown to be unnecessary for supernatant fractions. The procedure for assaying xanthine oxidase has already been described (Reid *et al.* 1956*a*).

Nucleotidase. There is evidence that liver cytoplasm contains a nucleotidase which has optimum activity near pH 6·3 and may be distinct from 'acid phosphatase' assayed at pH 5 with glycerophosphate as substrate (Goodlad & Mills, 1957; J. T. Nodes & E. Reid, unpublished work). Liver nucleotidase activity is apparently located in particulate elements (Novikoff, Podber, Ryan & Noe, 1953; Sonnenschein & Kopac, 1955; Goodlad & Mills, 1957; de Lamirande & Allard, 1957); but no attempt was made to study its location in the present experiments, which were performed with whole cytoplasmic fractions. The substrate used in most of the nucleotidase assays was a neutralized solution containing a mixture of adenosine 2′- and 3′-phosphates (a gift from Dr R. J. C. Harris) (0·025 M) in 0·1 M-cacodylate buffer (pH 6·3) (de Duve, Berthet, Hers & Dupret, 1949) which, in a few experiments, contained 9 mM-$MgCl_2$; 0·4 ml. of this solution and 0·3 ml. of tissue suspension (equivalent to about 20 mg. of liver tissue) were incubated for 30 min. at 37° with shaking. The reaction was stopped by addition of 2·5 ml. of 8% (w/v) trichloroacetic acid solution, and the supernatant obtained after centrifuging was analysed for inorganic phosphate. Activity values obtained with adenosine 2′- and 3′-phosphates as substrate were in some instances checked with other substrates. With uridine 2′- and 3′-phosphates, used as described above, the incubation time was prolonged to 90 min. because the rate of decomposition was only about 20% of that found with adenosine phosphate. Since it was of interest, in another connexion, to ascertain the effect of adrenalectomy on the rate of decomposition of nucleoside 5′-phosphates at pH 7·4, some assays were carried out with a solution of 0·0125 M-adenosine 5′-phosphate or uridine 5′-phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·4), usually containing 9 mM-$MgCl_2$; 0·4 ml. of this solution and 0·3 ml. of tissue suspension (equivalent to about 4 mg. of liver tissue) were incubated for 15 min. at 37° with shaking.

It is uncertain whether a single enzyme is concerned in the reactions now studied with these different substrates (cf. Christman, 1952). Moreover, the possible role of magnesium as an activator requires further study: preliminary experiments have suggested that there is some activation (about 30%) with Mg^{2+} ions present at a final concentration of 0·005 M, except perhaps with adenosine 5′-phosphate as substrate.

Adenosine deaminase, nucleoside phosphorylase and uricase. These enzymes were assayed by differential spectrophotometry at room temperature (21–22°) by the procedures of Schneider & Hogeboom (1952) based on those of Kalckar (1947*a*); the activity of each tissue sample, at pH 7·4, was assessed from the rate of change of extinction (determined graphically) at the appropriate wavelength. Uricase, which is known to occur in particles (Schneider & Hogeboom, 1952; Novikoff *et al.* 1953; de Duve *et al.* 1955) or possibly adsorbed on particles (London & Hudson, 1956), was determined in whole cytoplasmic fractions; but adenosine deaminase and nucleoside phosphorylase were determiued in supernatant fractions in which virtually all the cytoplasmic activity is located, as shown by Schneider & Hogeboom (1952) and as now confirmed for nucleoside phosphorylase.

Nucleoside phosphorylase was assayed with inosine (which, together with adenosine, was obtained from L. Light and Co., Slough, Bucks) as substrate, in the presence of KCN and of an excess of purified xanthine oxidase which converted the hypoxanthine (formed from inosine by the tissue phosphorylase) into uric acid. The activity now found with normal rats was three to four times that observed by Schneider & Hogeboom (1952) with mice, whereas the adenosine deaminase activity was of the same order as that which they observed although, in the present experiments, the concentration of adenosine was halved and a different buffer was used (tris buffer in place of glycylglycine).

Table 1. *Liver enzymes in relation to hormonal status*

Activities are expressed as μmoles of substrate decomposed/min. by an amount of tissue corresponding to 100 g. body wt. In this Table and in Table 2, the standard error is given after each mean difference, and the number of degrees of freedom, and (where appropriate) the probability that the difference could be due to chance, are given parenthetically.

Enzyme	Control rats: mean value	Experimental rats: difference from corresponding control rats		
		Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized
Nucleotidase (no added Mg^{2+} ions)	6·1	−1·8±0·24 (3; $P<1\%$)	−1·1±0·57 (3)	−0·8±0·86 (5)
Adenosine deaminase	1·8	+0·1±0·15 (4)	+0·8±0·46 (3)	−0·1±0·34 (7)
Nucleoside phosphorylase	39	0±6·4 (4)	−8±8·4 (3)	+7±6·3 (5)
Uricase	0·85	+0·1±0·22 (4)	−0·1±0·28 (3)	−0·3±0·09 (6; $P<2·5\%$)
Acid phosphatase, 'total' activity: Mitochondrial fraction	27	—	—	−3±2·3 (4)
Acid phosphatase, 'total' activity: Supernatant fraction	4·1	—	—	+0·7±0·50 (3)
Acid phosphatase, 'free' activity of mitochondrial fraction	0·7	—	—	−0·14±0·067 (4)

Uricase was assayed in the presence of a lower concentration of substrate (0·013 mM) than that used by Schneider & Hogeboom (1952) and by de Duve *et al.* (1955); with the final concentration used by the latter authors the extinction coefficient was unduly high. However, the activity now observed was of the same order as that of de Duve *et al.*, although only one-third of that found for rat liver by Schneider & Hogeboom (1952). It was found to be advantageous to add the substrate in a small volume (say 0·01 ml.) after 'pre-incubation' of the diluted tissue sample for 1–2 hr. at room temperature; without this pre-incubation the blanks (without substrate) did not give consistently stable readings, possibly because of changes in the aggregation of cytoplasmic particles.

Acid phosphatase. Assays were performed according to de Duve *et al.* (1955), at pH 5 with β-glycerophosphate as substrate. It was verified that the addition of Mg^{2+} ions did not enhance the activity. The tissue samples were usually frozen and thawed eight times before assay, as with nuclease assays; but assays for 'free' activity were carried out with freshly isolated mitochondrial fractions as described by de Duve *et al.*

Magnesium. The colorimetric method of Orange & Rhein (1951) was used.

Hypoxanthine plus xanthine. Kalckar (1947*b*) remarked that these compounds could be estimated in tissue by his spectrophotometric procedures, but did not actually study tissue samples. The extraction method used for nucleotides by Hurlbert & Potter (1954) and by Reid & Stevens (1957) has now been adapted for the assay of the hypoxanthine or xanthine or both present in liver, the assay being performed by differential spectrophotometry (Kalckar, 1947*b*), whereby the uric acid formed under the action of added xanthine oxidase is measured.

Rapidly excised liver was ground to a powder in the frozen state, extracted with cold perchloric acid solution, and the extract neutralized with KOH, as described by Hurlbert & Potter (1954). The phenol red used as internal indicator did not interfere in the final assay, but the trace of perchlorate remaining in solution after removal of the $KClO_4$ precipitate was sufficient to inhibit the xanthine oxidase completely. This difficulty was overcome by brief treatment of the neutralized extract with Dowex-1 resin (Cl^- form). It was then found that the xanthine oxidase was fully active, as shown by recovery experiments with added xanthine.

When the solution (3 ml.) in the cuvette contained an amount of extract equivalent to 0·2–0·3 g. of liver, the increase in extinction at 292 mμ due to formation of uric acid was readily measurable. Kalckar (1947*b*) suggests that the actual disappearance of hypoxanthine and of xanthine can be followed by measuring changes in extinction at 248 and 270 mμ respectively. The extinction of the liver extract was too high to permit accurate readings even at 270 mμ, but such values as could be obtained at 270 mμ suggested that xanthine rather than hypoxanthine was the source of the uric acid formed in the assay.

RESULTS

Liver constituents as affected by hypophysectomy, growth-hormone treatment and adrenalectomy

The results of the enzymic assays are summarized in Table 1. In general, the enzymes studied were little affected by hypophysectomy or adrenalectomy, and the levels in hypophysectomized rats were not significantly changed by administration of growth hormone.

Nucleotidase. This activity, assayed with adenosine 2′- and 3′-phosphates in the absence of magnesium, fell by 30% after hypophysectomy (Table 1). A few experiments carried out with

added magnesium, or with other substrates as indicated in the Experimental section, gave results in accord with those tabulated.

Adenosine deaminase and nucleoside phosphorylase. It is evident that there were no significant changes in the activities of these enzymes.

Uricase. A significant decrease (34 %) was found after adrenalectomy, although not after hypophysectomy.

Acid phosphatase. This enzyme, which may be unconnected with nucleic acid catabolism, was studied for reasons indicated in the Discussion. Mathies, Gaebler & Palm (1949) found that the acid-phosphatase activity of whole liver is unaffected by hypophysectomy, whether or not growth hormone is given. Adrenalectomy is also without effect (Kochakian & Vail, 1944), as now confirmed by the values (Table 1) for the 'total' activities of mitochondrial fractions (which contain the bulk of the cytoplasmic activity) and of supernatant fractions and for the 'free' activity of mitochondrial fractions.

Magnesium. Since Mg^{2+} ions may enhance the activity of several of the liver enzymes studied in this Laboratory, namely ATP-ase, nucleotidase and acid ribonuclease (although recent experiments (J. T. Nodes & E. Reid, unpublished work) have not confirmed the claim of de Lamirande, Allard, da Costa & Cantero (1954) that Mg^{2+} ions activate ribonuclease with RNA as substrate), it was desirable to verify that the amount of magnesium in liver does not depend on hormonal status. Analyses now performed on liver fractions have shown that hypophysectomy (with or without growth-hormone treatment) and adrenalectomy have no marked influence on the level or distribution of magnesium in liver cytoplasm. Normal cytoplasm was found to contain about 200 μg. of magnesium/g. of liver, half of which was in the supernatant fraction and the remainder divided between the mitochondrial and microsomal fractions.

Xanthine. Since the level of xanthine oxidase falls after adrenalectomy (Reid *et al.* 1956*a*), it was of interest to ascertain whether its substrate showed an increased endogenous level. However, four determinations on intact rats gave quite consistent values, averaging 0·085 μmole/g. of liver; for corresponding adrenalectomized rats the average value was 0·09 μmole/g. As already indicated, it is probable that the compound estimated was xanthine rather than hypoxanthine.

Changes in liver constituents after adrenalectomy, in relation to time after operation

Effects of adrenalectomy on the incorporation of injected precursors into protein and RNA were investigated by Reid & Stevens (1957) with respect to their time of onset after operation. Other effects of adrenalectomy have now been studied from this aspect, with the results shown in Fig. 1.

Deoxyribonucleic acid and deoxyribonuclease. Reid (1956) found a rise in liver DNA in female adrenalectomized rats. This rise has been confirmed in the present experiments with male rats, and is evidently a delayed effect of adrenalectomy, whereas the rise in cytoplasmic DNA-ase (Stevens & Reid, 1956) has already commenced 3 days after operation. It has also been found that the rise in the amount of DNA-ase in the supernatant fraction is already significant ($P < 5$ %) 3 days after operation; this rise is not illustrated in Fig. 1, the values for normal liver being so low that the increases cannot be accurately expressed as percentages of the control values.

Ribonucleic acid and ribonuclease. The curve for supernatant-fraction RNA is reproduced from Fig. 6 of the preceding paper in this series (Reid & Stevens, 1957). It will be noted that the rise in the RNA-ase of the whole cytoplasmic fraction or of the supernatant fraction (Stevens & Reid, 1956), unlike the rise in RNA, is a delayed effect of operation.

These and other hormonal effects were studied in rats which had been fasted overnight before autopsy. The possibility that the rise in the amount of RNA in the supernatant fraction after adrenalectomy might be manifest only in fasted rats has now been checked, and the rise has been found to occur also in fed rats.

Xanthine oxidase and uricase. It is evident that the decrease in xanthine oxidase which was found by Reid *et al.* (1956*a*) occurs gradually after

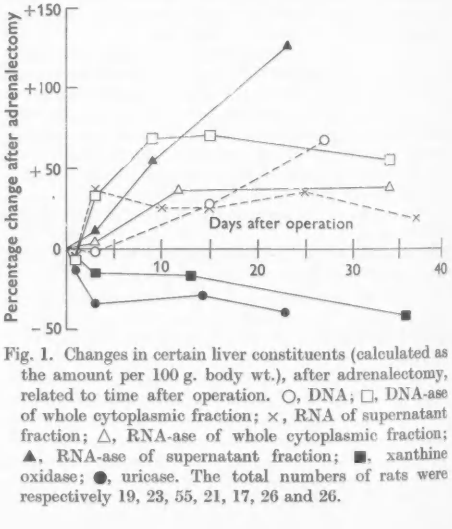

Fig. 1. Changes in certain liver constituents (calculated as the amount per 100 g. body wt.), after adrenalectomy, related to time after operation. ○, DNA; □, DNA-ase of whole cytoplasmic fraction; ×, RNA of supernatant fraction; △, RNA-ase of whole cytoplasmic fraction; ▲, RNA-ase of supernatant fraction; ■, xanthine oxidase; ●, uricase. The total numbers of rats were respectively 19, 23, 55, 21, 17, 26 and 26.

Table 2. *Kidney nucleases and deoxyribonucleic acid in relation to hormonal status*

Nuclease activities are expressed as μmoles of mononucleotide liberated/min. by an amount of tissue corresponding to 100 g. body wt., it being assumed that the acid-soluble digestion products are entirely mononucleotides (de Duve *et al.* 1955). DNA levels are expressed as mg. of DNA in an amount of tissue corresponding to 100 g. body wt.

		Control rats: mean value	Experimental rats: difference from corresponding control rats		
			Hypophysectomized	Hypophysectomized +growth hormone	Adrenalectomized
DNA-ase	Whole kidney	0·45	+0·28±0·164 (3)	+0·32±0·126 (3)	+0·095±0·056 (4)
	Supernatant fraction	0·22	+0·135±0·054 (5)	+0·14±0·134 (4)	+0·13±0·078 (4)
RNA-ase	Whole kidney	3·66	+1·59±0·636 (3)	+1·16±0·403 (3)	+0·47±0·519 (4)
	Supernatant fraction	1·69	+0·58±0·218 (5; $P<5\%$)	+0·67±0·156 (4; $P<2·5\%$)	+0·62±0·327 (4)
DNA		0·62	+0·06±0·103 (5)	+0·10±0·162 (4)	+0·21±0·063 (5; $P<2·5\%$)

operation, whereas the decrease in uricase is more abrupt.

Other effects of adrenalectomy. (These are not illustrated in Fig. 1.) Reid *et al.* (1956*a*) found that adrenalectomy led to a decrease in the ATP-ase activity, assayed in the presence of Mg^{2+} ions, of the whole cytoplasmic fraction and of the microsomal fraction. It has now been found that these changes are not apparent 3 days after operation, nor is the classical effect of adrenalectomy in increasing the weight of the thymus manifest at 3 days. Of the other changes in organ weights after adrenalectomy, namely a 10% increase in heart weight and a 20% increase in lung weight (unpublished observations in this Laboratory), only the latter change is in evidence at 3 days.

Kidney constituents as affected by hypophysectomy, growth-hormone treatment and adrenalectomy

Table 2 shows that DNA-ase and RNA-ase activities, assayed at pH 5 as in the previous experiments with liver, tended to rise after hypophysectomy (with or without growth-hormone treatment) or adrenalectomy, both in whole kidney and in the supernatant fraction. However, the rise was significant only in the supernatant-fraction RNA-ase of the hypophysectomized rats. In contrast with liver supernatant fractions, which normally contain little nuclease activity, the kidney supernatant fractions from the control rats were almost half as active as the whole kidney. This high activity was not attributable merely to the use of frozen-kidney tissue; for control rats the mean ratio of supernatant-fraction RNA-ase activity to whole-homogenate activity was 0·50 in seven experiments with frozen kidney, as compared with 0·37 in three experiments with fresh kidney. It is also evident from Table 2 that kidney DNA was increased after adrenalectomy, although not after hypophysectomy. From previous work (Reid, 1956) it can be concluded that there are no significant changes in RNA levels in fractions obtained from kidney cytoplasm.

DISCUSSION

The possibility that nucleases are anabolic rather than catabolic *in vivo* has been discussed by Stevens & Reid (1956). With DNA-ase, it might appear that this possibility is supported by the present observation that the rise in liver DNA-ase after adrenalectomy precedes the rise in DNA (Fig. 1). Since, however, the increase in kidney DNA occurring after adrenalectomy is not accompanied by as marked a rise in DNA-ase as occurs in liver, it is questionable whether DNA increases by virtue of an increase in DNA-ase. The possibility that acid RNA-ase has an anabolic role *in vivo* now appears remote. The precursors of liver RNA appear to be nucleoside 5′-phosphates (Hurlbert & Potter, 1954; Herbert, Potter & Hecht, 1957), rather than the 2′- or 3′-phosphates which are formed under the action of acid RNA-ase (J. T. Nodes & E. Reid, unpublished work). Moreover, the rise in liver RNA-ase after adrenalectomy occurs later than the rises in supernatant-fraction RNA (Fig. 1) and in the rate of synthesis of this RNA (Reid & Stevens, 1957). Although it is probable that RNA-ase is a catabolic enzyme, it should not be concluded that the rise in RNA-ase reflects an increase in catabolism of supernatant-fraction RNA consequent on the increase in synthesis. The absence of a continued increase in supernatant-fraction RNA in rats adrenalectomized more than 3 days previously is attributable primarily to a diminution in the elevated rate of synthesis, rather than to an increase in catabolism (Reid & Stevens, 1957). It should also be pointed out that untreated hypophysectomized rats show increased RNA-ase levels in liver (Stevens & Reid, 1956) and kidney with

no increase in the amount of RNA in any of the fractions derived from the cytoplasm (Reid, 1956).

One difficulty in interpreting the changes in nuclease levels is that most of the activity is normally 'bound' in particles which are deliberately ruptured for the purpose of assay (de Duve *et al.* 1955). A change in the normally low nuclease activity of the supernatant fraction may be of more physiological significance than a change in the bound activity of the particles. It may be recalled that the rises in cytoplasmic nuclease activity after hypophysectomy or adrenalectomy were in part due to exaggerated rises in supernatant-fraction activity (Stevens & Reid, 1956; present paper, Fig. 1), and that increases in the RNA-ase and acid-phosphatase activities of the supernatant fraction (but not of particulate fractions) have been observed during carcinogenesis (Allard, 1955; Reid & O'Neal, 1956; cf. de Duve, 1957). Stevens & Reid (1956) suggested that hypophysectomy or adrenalectomy may increase the fragility or permeability of the nuclease-containing particles with a consequent increase in supernatant-fraction activity. These particles are considered by de Duve *et al.* (1955) to be identical with the particles ('lysosomes') which contain acid phosphatase; this conclusion is based partly on the close parallelism between the liberation of acid phosphatase and that of RNA-ase or DNA-ase (all three enzymes being liberated in soluble form) when the lysosomes are disrupted. However, acid-phosphatase activity, in contrast with nuclease activity, is not increased in the supernatant fraction after adrenalectomy; moreover, the lysosome-containing fraction shows no increase in 'free' acid-phosphatase activity (Table 1) such as might occur (de Duve *et al.* 1955) if the lysosomes from adrenalectomized rats were abnormally fragile. The data for acid phosphatase therefore suggest that supernatant-fraction nuclease activity does not increase merely because of non-specific damage to lysosomes.

In general it is difficult to interpret the changes in enzyme levels now observed because it is uncertain which, if any, of these enzymes are 'limiting' in the chain of nucleic-acid catabolism. This chain may be represented for the adenylic acid of RNA as shown in Scheme (I) (Christman, 1952), taking account of a recent finding (J. T. Nodes & E. Reid, unpublished work) that the acid RNA-ase of liver liberates purine as well as pyrimidine mononucleotides from RNA:

RNA → adenosine 2′- or 3′-phosphate (RNA-ase) → adenosine (nucleotidase) → inosine (adenosine deaminase) → hypoxanthine (nucleoside phosphorylase) → xanthine (xanthine oxidase) → uric acid (xanthine oxidase) → allantoin (uricase).

(I)

It is not suggested that the reactions shown in scheme I are irreversible, or that alternative pathways do not exist in liver, but it should be pointed out that liver does not readily deaminate adenosine monophosphates as distinct from adenosine (Conway & Cooke, 1939). Umbreit & Tonhazy (1951*a*) have reported that the deamination of adenosine 5′-phosphate by kidney is increased after adrenalectomy, but the possibility that this reaction proceeds by way of adenosine was not excluded.

In assessing whether any of these enzymes are limiting, one approach is to compare their activities as found in the present assays. From the values for control rats (Table 3 in Reid *et al.* 1956*a*; Table 2 in Stevens & Reid, 1956; Table 1 in the present paper), it appears that the activities of xanthine oxidase, uricase and possibly RNA-ase are relatively low, whereas that of nucleoside phosphorylase is notably high. However, this approach disregards important factors, such as differences in assay conditions and possible spatial separations of enzymes from one another and from their substrates within the cytoplasm.

Another approach might be to ascertain which enzymes are most prone to vary with changes in the physiological state of the animal. The levels of RNA-ase, xanthine oxidase and uricase change with changes in hormonal status as now studied, and also in carcinogenesis (Allard, 1955; de Lamirande & Allard, 1957; Bergel, Bray, Haddow & Lewin, 1957; Reid & Lewin, 1957). Moreover, xanthine oxidase is markedly influenced by diet (Richert & Westerfeld, 1950) or by exogenous xanthine (Feigelson, Feigelson & Wood, 1954).

These considerations suggest that the enzymes which are most likely to be limiting are RNA-ase, uricase or, as Bergel *et al.* (1957) have postulated, xanthine oxidase. However, with xanthine oxidase the simplest interpretation of the absence of an accumulation of xanthine or hypoxanthine in the livers of adrenalectomized rats is that this enzyme is not limiting. Indeed, in rats fed on a low-protein diet so as to cause a marked depletion of xanthine oxidase in the liver, Bass, Tepperman, Richert & Westerfeld (1950) found that there was undiminished excretion of uric acid and allantoin (cf. Williams, Feigelson & Elvehjem, 1950; Richert & Westerfeld, 1957).

The present finding that uricase activity is already markedly decreased 3 days after adrenalectomy apparently accords well with the conclusion of Romanoff & Hunt (1954) that allantoin formation during fasting is decreased 4 days after adrenalectomy; after hypophysectomy it is also decreased, which is in apparent contradiction with the present findings. Since, however, these authors found that allantoin formation during re-feeding

was undiminished in adrenalectomized rats, it would appear that neither xanthine oxidase nor uricase was limiting. Further evidence against the possibility that uricase is limiting comes from the finding of Williams *et al.* (1950) that its level falls by 35% after protein deprivation with no fall in allantoin excretion.

The study of time of onset of effects after a change in hormonal status of animals may indicate not only interrelationships between processes (cf. Reid & Stevens, 1957), but also points of action of hormones, if the reasonable supposition is made that processes which change at an early stage lie close to, although not necessarily at, primary points of hormone action. Changes which are already evident 3 days after adrenalectomy include not only increased synthesis of supernatant-fraction RNA, increased DNA-ase activity and decreased uricase activity, but also (Umbreit & Tonhazy, 1951*b*) decreased oxidation of proline by kidney.

SUMMARY

1. Enzymes concerned in nucleic acid catabolism have been studied with liver cytoplasm and, for nucleases, with kidney obtained from untreated hypophysectomized rats, hypophysectomized rats treated with growth hormone (which did not affect any of the processes studied) and adrenalectomized rats.

2. Nucleotidase activity fell after hypophysectomy, and uricase activity fell after adrenalectomy. The activities of adenosine deaminase and purine nucleoside phosphorylase showed no changes, nor were there any marked changes in the amount or distribution of magnesium in liver cytoplasm.

3. On the third day after adrenalectomy, changes were already evident (in liver) in uricase, supernatant-fraction ribonucleic acid and deoxyribonuclease (both whole-cytoplasmic fraction and supernatant fraction), whereas changes in deoxyribonucleic acid, ribonuclease (both whole-cytoplasmic fraction and supernatant fraction) and xanthine oxidase tended to occur later.

4. Kidney tissue showed a rise in the ribonuclease activity of the supernatant fraction after hypophysectomy, and a rise in deoxyribonucleic acid content after adrenalectomy.

5. The levels of acid phosphatase and xanthine in liver, studied to assist in interpreting these results, were not markedly affected by adrenalectomy.

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Polyol Dehydrogenase of the Silkworm

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It is becoming increasingly evident that a number of important metabolic events take place in the haemolymph of insects. Examples of the reactions catalysed by enzymes of insect haemolymph are hydrolysis of carbohydrates, fat and proteins, the oxidation of tyrosine and the decomposition of hydrogen peroxide (see review by Buck, 1953). Transaminase has also been detected (Bheemeswar & Sreenivasaya, 1952), as well as a specific phosphatase (Faulkner, 1955).

Recent studies from this laboratory have indicated that the haemolymph of the silkworm, *Bombyx mori* L., contains triphosphopyridine nucleotide-linked dehydrogenases which oxidize L-malate and reduce sugar phosphates (Faulkner, 1956*a*, *b*). It has now been found that hydroxyaldehydes and other carbonyl compounds are reduced by the same enzyme preparation and an account of these studies is given here.

MATERIALS AND METHODS

Dialysed silkworm haemolymph. This was obtained from fifth-instar larvae as described previously (Faulkner, 1956*a*).

Extracts of silkworm tissues. These were obtained by grinding the tissue (0·3–1 g.) with 3 ml. of water at 0° in a motor-driven glass homogenizer. The mixture was centrifuged at 10 000 *g* for 5 min. at 0° and the supernatant was collected. Enzyme activities of the extracts were expressed as units/mg. of protein.

Chemicals. Glycolaldehyde was prepared by the procedure of Powers, Tabakoglu & Sable (1955), glyoxylic acid according to Weinhouse & Friedmann (1951) and D- and L-glyceraldehyde by the method of Perlin & Brice (1956). D-Erythrose and D-threose were gifts from Dr A. S. Perlin, and L-erythrulose from Dr N. Yattrie, both of the Prairie Regional Laboratories, Saskatoon. Other chemicals were purchased as follows: pentoses, sugar acids and uronic acids (Pfanstiehl Chemical Co., Waukegan, Ill., U.S.A.); dihydroxyacetone, DL-glyceraldehyde and L-sorbose (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); glucose 6- and ribose 5-phosphates (Schwarz Laboratories Inc., New York, U.S.A.); reduced triphosphopyridine nucleotide (TPNH) and reduced diphosphopyridine nucleotide (DPNH) (Sigma Chemical Co., St Louis, Mo., U.S.A.); D-galactose, D-mannose (Difco Inc., Detroit, Mich., U.S.A.); and methylglyoxal (Bios Chemical Co., New York, U.S.A.); the remaining chemicals were purchased from Fisher Scientific Co., Toronto, Ontario, Canada.

The sugar acids supplied as lactones were converted into the sodium salts by heating with dil. NaOH. Materials used as test substrates were neutralized by addition of HCl or NaOH.

Spectrophotometric determination of dehydrogenase. The standard test system used in the assay of dehydrogenase activity contained the following in a final volume of 2·5 ml.: 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·5 (18 mM); $MgSO_4$ (4 mM); TPNH (0·1 mM); substrate and enzyme. Cuvettes (1 cm. light path) containing all the components except the substrate were incubated at room temperature (20°) in a Beckman DU spectrophotometer. Optical-density readings were taken at 340 mμ and after a 2 min. equilibration period the substrate (final concn. 2 mM) was added and readings were taken every 30 sec. The blank cell used in the assay did not contain substrate or TPNH. Optical density was plotted against time of incubation and the enzyme activity was calculated from the slope of the initial linear portion of the curve. One unit of enzyme was defined as the amount necessary to reduce the optical density by 0·01/min. Incubations did not normally continue more than 5 min. after the addition of substrate and during this period no appreciable heating of the samples was observed.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), and polyhydric alcohols by the method of West & Rapoport (1949) with the modifications reported previously (Faulkner, 1956*b*).

EXPERIMENTAL AND RESULTS

Reduction of DL-*glyceraldehyde*

In the presence of DL-glyceraldehyde and buffer (pH 7·5) dialysed silkworm haemolymph catalyses the oxidation of TPNH. The reaction does not

occur in the absence of dialysed haemolymph nor when the latter is replaced by heated enzyme, nor does it take place when DPNH is substituted for TPNH. The rate of oxidation of TPNH in the standard test system is proportional to the concentration of enzyme (Fig. 1). These observations indicate that a TPN-linked dehydrogenase which reduces DL-glyceraldehyde is present in the haemolymph of the silkworm. The enzyme, which is active in the range pH 4·0–9·5, has no pronounced maximum between these pH limits.

Although magnesium was included in the standard incubation mixture used to study the reduction of DL-glyceraldehyde, an absolute requirement for the cation could not be shown with either dialysed haemolymph or haemolymph that had been fractionated by treatment with ammonium sulphate and calcium phosphate. Sodium ethylenediaminetetra-acetate (10 mM) had no effect on the rate of reduction of DL-glyceraldehyde in the standard test system with dialysed haemolymph.

It is possible to estimate the amount of DL-glyceraldehyde that can be reduced in 1 hr. by 1 ml. of silkworm haemolymph containing 30 mg. of protein/ml. The specific activity of the glyceraldehyde-reducing enzyme in whole or dialysed haemolymph is about 1·3 units/mg. of protein. If the value of $6{\cdot}22 \times 10^6$ cm.^{2}/mole is taken for the extinction coefficient of TPNH (Horecker & Kornberg, 1948), it may be calculated that 1 ml. of haemolymph could catalyse the reduction of 0·85 mg. of DL-glyceraldehyde/hr. under the standard test conditions.

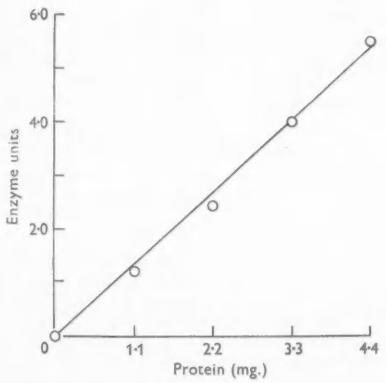

Fig. 1. Relationship between rate of reduction of DL-glyceraldehyde and enzyme concentration. The standard test system was used, with DL-glyceraldehyde (2 mM) as substrate and dialysed haemolymph as enzyme. The total volume incubated was 2·5 ml.

Substrate specificity

A number of sugars and other carbonyl compounds were tested to determine whether they are reduced by a triphosphopyridine nucleotide (TPN)-linked enzyme in silkworm haemolymph. The incubation mixture contained TPNH, Mg^{2+} ions and buffer (pH 7·5) in addition to the enzyme and test substrate. The results are expressed as the rate at which the test substrate was reduced compared with that for D-glyceraldehyde, which was arbitrarily fixed at 100. The concentration of test substrate and of D-glyceraldehyde was 4 mM. Comparative reduction rates with a number of sugars and sugar acids are given in Table 1. It will be seen that glycolaldehyde, D- and L-glyceraldehyde and the tetroses are all readily reduced. In contrast, the pentoses are reduced only slowly and the hexoses not at all. The rate with L-glyceraldehyde is about half that obtained with D-glyceraldehyde. The enzyme has a higher affinity for D- than for L-glyceraldehyde since the Michaelis constant

Table 1. *Rates of reduction of sugars compared with the rate for* D-*glyceraldehyde*

The standard test conditions were used with dialysed haemolymph and with the substrates present at 4 mM. For details see text.

Class	Test substrate	Relative rate of reduction
Diose	Glycolaldehyde	47
Trioses	D-Glyceraldehyde	100
	L-Glyceraldehyde	49
	DL-Glyceraldehyde	100
	Dihydroxyacetone	5
Tetroses	D-Erythrose	131
	D-Threose	104
	L-Erythrulose	19
Pentoses	D-Arabinose	5
	L-Arabinose	12
	D-Ribose	9
	D-Lyxose	2
	D-Xylose	19
Hexoses	D-Glucose	0
	D-Galactose	0
	D-Mannose	0
	D-Fructose	0
	L-Sorbose	0
Sugar acids	Gluconic	0
	Galactonic	0
	Gulonic	0
Uronic acids	Glucuronic	89
	Galacturonic	50
Sugar phosphates	Ribose 5-phosphate	107
	Glyceraldehyde 3-phosphate + dihydroxyacetone phosphate mixture*	0

* Obtained by treatment of hexose diphosphate with aldolase.

for D-glyceraldehyde is 0·066 mM and for L-glyceraldehyde is 2·7 mM in dialysed haemolymph, as determined by the method of Lineweaver & Burk (1934).

The rate of reduction of dihydroxyacetone is only 5% of that obtained with D-glyceraldehyde, which indicates that the insect enzyme is quite distinct from the glycerol dehydrogenases of *Escherichia coli* (Asnis & Brodie, 1953), *Aerobacter aerogenes* (Burton & Kaplan, 1953; Burton, 1955) and *Pseudomonas salinaria* (Baxter & Gibbons, 1954) with which the reduction of dihydroxyacetone is the faster reaction.

Sugar acids, e.g. gluconic and galactonic acids, are not reduced in this system, but the uronic acids, which retain their carbonyl group at the $C_{(1)}$ position, are readily reduced.

In Table 2 the rates of reduction of some $C_{(1)}$–$C_{(4)}$ carbonyl compounds are compared with that of D-glyceraldehyde. The unsubstituted simple aldehydes and ketones, e.g. formaldehyde and acetone, are not reduced nor are the keto acids, glyoxylic and pyruvic acids. However, the unsaturated aldehyde acrolein, and aldehydes which carry hydroxyl substituents, are reduced.

The dicarbonyl compounds glyoxal, methylglyoxal and dimethyglyoxal are reduced at rates approaching those obtained with an equimolar amount of D-glyceraldehyde. It is interesting that acetylacetone, in which the two carbonyl groups are separated by a $-CH_2$ group, is not reduced.

Identification of end products

Glycerol formation. Sufficient quantities of the product of reduction of DL-glyceraldehyde for analytical purposes were obtained by providing for the continuous formation of TPNH in the incubation mixture. This was achieved by incubating DL-glyceraldehyde in the presence of L-malate and a small quantity of TPN. In this mixture TPN was reduced by the 'malic' enzyme of the haemolymph (Faulkner, 1956*a*) and the following coupled reaction occurred with the 'aldehyde-reducing' enzyme:

$$\text{L-Malate} + \text{TPN}^+ \rightarrow \text{pyruvate} + CO_2 + \text{TPNH}$$

$$\text{TPNH} + \text{DL-glyceraldehyde} \rightarrow \text{'reduced glyceraldehyde'} + \text{TPN}^+$$

Two tubes were each filled with the following mixture: tris buffer, pH 7·5, 20 μmoles; $MgSO_4$, 5 μmoles; L-malate, 30 μmoles; TPN, 0·25 μmole; DL-glyceraldehyde, 10 μmoles; dialysed haemolymph, 0·5 ml. The total volume was 2·05 ml. The enzyme in one of the tubes was inactivated by heating the contents for 5 min. at 100°. Both tubes were then incubated for 3 hr. at 30°. The protein of the second tube was precipitated by heating and both tubes were centrifuged. A volume (1·5 ml.) of each supernatant was treated with 2 ml. of 2:4-dinitrophenylhydrazine (0·1% in 2N-HCl) and the mixture was incubated at 30° for a further 30 min. It was then centrifuged at high speed and the supernatant dried over silica gel *in vacuo*. The residue was suspended in 0·2 ml. of water and samples (20 μl.) were applied to Whatman no. 1 papers for ascending chromatography. Papers were developed for 18 hr. in the following solvents: (i) *n*-butanol–acetic acid–water (25:6:25, by vol., upper layer; Woiwod, 1949); (ii) *n*-butanol–ethanol–water (4:1:5, by vol., upper phase; Hackman & Trikojus, 1952); and (iii) phenol (80%, w/v). After development, polyhydric alcohols were detected by spraying the paper with periodate followed by a benzidine reagent (test 1, Cifonelli & Smith, 1954).

The incubated mixture was found to contain a substance having the same R_F as glycerol in all three developing solvents. No glycerol was found in the control. It is concluded that glycerol is formed when TPNH reduces DL-glyceraldehyde in the presence of dialysed haemolymph.

Quantitative measurements on the formation of glycerol were made on mixtures of DL-glyceraldehyde, TPN, L-malate and dialysed haemolymph. Samples of the mixtures which had been incubated and deproteinized were analysed by paper electrophoresis at pH 9 in a borate buffer (0·05M) for 4 hr. under a potential gradient of 10 v/cm. Under these conditions DL-glyceraldehyde and glycerol could be separated and the glycerol was eluted from the paper. The results given in Table 3 show that the amount of glycerol produced is proportional to the time of incubation.

Erythritol formation. The formation of erythritol was demonstrated when L-malate (30 μmoles), TPN (0·25 μmole)

Table 2. *Rates of reduction of carbonyl compounds compared with the rate for D-glyceraldehyde*

The standard test conditions were used with dialysed haemolymph and with substrates present at 4 mM.

Class	Name	Relative rate of reduction
Monocarbonyl	Formaldehyde	0
	Acetaldehyde	0
	Acetone	0
	Pyruvic acid	0
	Acrolein	72
	D-Glyceraldehyde	100
	Aldol	37
	Acetoin	29
	Glyoxylic acid	0
Dicarbonyl	Glyoxal	86
	Methylglyoxal	93
	Dimethylglyoxal	98
	Acetylacetone	0

Table 3. *Production of glycerol in dialysed haemolymph*

Incubation medium: tris buffer, pH 7·5, 20 mM; $MgSO_4$, 5 mM; L-malate, 10 mM; TPN, 0·05 mM; DL-glyceraldehyde, 2 mM; dialysed haemolymph, 0·2 ml. Tubes, containing 1 ml., were incubated for the stated time at 30°. Glycerol was separated as described in the text and determined by the method of West & Rapoport (1949).

Incubation time (min.)	Glycerol found (μmoles)
0	0
30	0·34
60	0·74
90	1·07

and D-erythrose (10 μmoles) were incubated in dialysed haemolymph (0·5 ml.), tris buffer, pH 7·5 (20 μmoles) and $MgSO_4$ (5 μmoles) for 3 hr. at 30°. The procedure for working up the incubated mixtures before chromatography was the same as that used for glycerol. Erythritol was resolved by chromatography in the following solvents: (i) *n*-butanol–ethanol–water (4:1:5, by vol., upper phase; Hackman & Trikojus, 1952); (ii) *n*-butanol–pyridine–water (4:1:1, by vol.; Hockenhull, 1953); (iii) phenol (80%, w/v). No erythritol was present in the control mixture which was inactivated at the beginning of the experiment.

Ethylene glycol formation. Glycolaldehyde, L-malate and TPN were incubated with dialysed haemolymph, and the mixture was inactivated and concentrated as described in the section on glycerol formation. Samples of the supernatant were applied to filter paper for development in *n*-butanol–ethanol–water (4:1:5, by vol., upper phase; Hackman & Trikojus, 1952). 'Marker' quantities (0·1 μmole) of ethylene glycol were applied close to the borders of the chromatogram. After development, the strips bearing the 'marker' were cut from the paper and were treated with the periodate–benzidine spray. Portions of the developed chromatogram were then eluted at the level of ethylene glycol as determined on the 'marker' strips and the polyhydric alcohol content of the eluate was measured colorimetrically. In a typical experiment in which glycolaldehyde (10 μmoles), L-malate (30 μmoles) and TPN (0·25 μmole) were incubated with dialysed haemolymph, tris buffer, pH 7·5 (20 μmoles) and $MgSO_4$ (5 μmoles) for 3 hr. at 30°, 0·49 μmole of ethylene glycol was formed, none being detected in a control inactivated at the beginning of the experiment. Under similar incubation conditions DL-glyceraldehyde (10 μmoles) formed 3·42 μmoles of glycerol.

Reversibility

At pH 7·5 the equilibrium of the polyol dehydrogenase in silkworm haemolymph favours the complete reduction of added aldehydes, e.g. glyceraldehyde or glycolaldehyde, and it was not possible to demonstrate the oxidation of glycerol or ethylene glycol by incubating them with TPN and dialysed haemolymph. However, at pH 8·5 and at a high substrate concentration (M), several polyhydric alcohols reduce TPN. Of the compounds tested, 2:3-butanediol was most rapidly oxidized and propylene glycol and glycerol were oxidized at much lower rates. At the same concentration (M) no oxidation of ethylene glycol or sorbitol was observed.

Comparison of the 'malic' enzyme and polyol dehydrogenase

It has been established that silkworm haemolymph contains a TPN-linked malic dehydrogenase (Faulkner, 1956*a*). It was therefore important to determine whether the oxidation of L-malate and polyhydric alcohols is catalysed by the same enzyme. Evidence that two enzymes are involved was obtained when the rates of oxidation of L-malate and propylene glycol separately and in mixtures were compared. In a typical incubation experiment the basic medium contained tris buffer, pH 8·5 (16 mM); $MgSO_4$ (2 mM); TPN (0·2 mM); dialysed haemolymph, 0·5 ml. The total volume was 2·5 ml. The rate of oxidation of L-malate (8 mM) was 0·028 optical density (*E*) unit/min., and with propylene glycol (M) was 0·015 *E* unit/min. and with a mixture of substrates was 0·041 *E* unit/min., which is almost the sum of the individual rates.

Factors affecting the reduction of ribose 5-phosphate and DL-glyceraldehyde in the haemolymph

The incubation conditions for studying the reduction of sugars and carbonyl compounds were the same as those used for reducing sugar phosphates (Faulkner, 1956*b*). Experiments were carried out to determine whether all these reductions are catalysed by the same enzyme. To this end, the rates of reduction of ribose 5-phosphate and DL-glyceraldehyde were compared under a number of experimental conditions, namely in mixtures of ribose 5-phosphate and DL-glyceraldehyde and in different protein fractions of the haemolymph. The effect of inhibitors on the reduction rates was also tested.

In a typical experiment with dialysed haemolymph, a mixture of DL-glyceraldehyde and ribose 5-phosphate (both 2 mM) was reduced at a rate of 0·0235 *E* unit/min., whereas the rate with DL-glyceraldehyde alone was 0·023 *E* unit/min. and with ribose 5-phosphate was 0·024 *E* unit/min. At this concentration both substrates saturated the enzyme. If two enzymes were involved the reduction rate of the mixture should have approached the sum of the rates obtained with the compounds present individually. A similar result was obtained when the rate of reduction of a mixture of galactose 6-phosphate and DL-glyceraldehyde was measured.

Reduction rates of DL-glyceraldehyde and ribose 5-phosphate were compared in whole blood, in dialysed haemolymph and in three protein fractions obtained by treating whole blood with ammonium sulphate. The results of these measurements are given in Table 4 and they indicate that, irrespective of the treatment of the haemolymph, the ratio between the reduction rates of the sugars lies within the limits 0·93–1·16, although there was a fourfold difference in specific activity between two of the samples (I and II).

Further evidence that ribose 5-phosphate and DL-glyceraldehyde are reduced by the same enzyme is provided by the finding that the reduction of both substances is inhibited by ethylene glycol, propylene glycol and glycerol (Table 5). The observed inhibition of the rate of oxidation of TPNH is not due to the production of more TPNH by oxidation of the polyhydric alcohols, since at pH 7·5 no reaction takes place with TPN (see the

Table 4. *Rates of reduction of* DL-*glyceraldehyde and ribose 5-phosphate in protein fractions of the haemolymph*

The standard test conditions were used, with buffer, pH 7·5, and DL-glyceraldehyde and ribose 5-phosphate at 2 mM.

Treatment	Rate of reduction (units/mg. of protein)		Ratio of rates (glyceraldehyde: ribose phosphate)
	Glyceraldehyde	Ribose phosphate	
Fresh haemolymph	1·10	1·01	1·09
Dialysed haemolymph	1·30	1·39	0·93
Fresh haemolymph treated with $(NH_4)_2SO_4$			
I 0–40% of saturation	0·50	0·52	0·96
II 40–65% of saturation	2·06	2·19	0·94
III 65–95% of saturation	2·22	1·91	1·16

Table 5. *Effect of polyhydric alcohols on reduction of* DL-*glyceraldehyde and ribose 5-phosphate*

The standard test system was used with dialysed haemolymph and with DL-glyceraldehyde and ribose 5-phosphate both at 2 mM.

Polyhydric alcohol (M)	Percentage inhibition	
	DL-Glyceraldehyde reduction	Ribose 5-phosphate reduction
Ethylene glycol	44	58
Propylene glycol	59	56
Glycerol	40	33

Table 6. *Distribution of polyol dehydrogenase in silkworm tissues*

The standard test conditions were used with DL-glyceraldehyde (2 mM) as substrate. Tissues were prepared as described in Methods.

Tissue	Dehydrogenase (units/mg. of protein)
Fifth-instar larval blood	1·30
Fifth-instar larval fat body	1·13
Fifth-instar larval gut	2·21
Pre-pupa, 2 days after spinning	0·22
Pupa, 10 days after spinning	0·27
Adult	0·25

section on reversibility). Glycerol did not inhibit the 'malic' enzyme of the haemolymph, which shows that the alcohol is not a general inhibitor of all TPN-linked dehydrogenases of the haemolymph.

Distribution of polyol dehydrogenase in the silkworm

Polyol dehydrogenase activity was determined in a number of larval tissues as well as in preparations obtained from pupae and adults (Table 6). The enzyme was detected in all the tissues tested. When expressed as units/mg. of protein, the values obtained with pupal and adult preparations were lower than those with larval tissues. With the larval tissues it is interesting that the highest enzyme activity occurs in the gut, which may indicate that much of the fat metabolism in the larva occurs in this tissue.

DISCUSSION

Bacterial extracts that oxidize glycerol to dihydroxyacetone have been obtained from *Acetobacter suboxydans* (Hauge, King & Cheldelin, 1955), *Escherichia coli* (Asnis & Brodie, 1953), *Pseudomonas salinaria* (Baxter & Gibbons, 1954), *Bacillus subtilis* (Wiame, Bourgeois & Lambion, 1954) and *Aerobacter aerogenes* (Burton & Kaplan, 1953). These enzymes require DPN as coenzyme and with each the equilibrium favours the oxidation of glycerol. However, liver extracts from rat or pig possess a DPN-linked glycerol dehydrogenase, the equilibrium of which favours the formation of glycerol from D- or L-glyceraldehyde (Wolf & Leuthardt, 1953; Leuthardt & Wolf, 1954). The extract from liver also reduces acetaldehyde, and Holzer, Schneider & Lange (1955) have suggested that both reductions are catalysed by liver alcohol dehydrogenase.

The enzyme of silkworm haemolymph has its equilibrium in favour of glycerol synthesis and reduces D-glyceraldehyde at least 20 times as rapidly as dihydroxyacetone. It differs from the liver glycerol dehydrogenase in that it requires TPN as coenzyme and does not reduce acetaldehyde; it does, however, reduce a number of substances almost as readily as D-glyceraldehyde: e.g. D-erythrose is reduced to erythritol and glycolaldehyde yields ethylene glycol. The end products formed by the reduction of the other carbonyl compounds have not been identified, but, by analogy, one would expect the carbonyl group of these substances to be reduced to the corresponding carbinol.

The reduction of D-glucuronate and D-galacturonate in silkworm haemolymph is interesting in view of the recent finding that these compounds

are also reduced by enzymes in rat liver (Publitz, Grollman & Lehninger, 1957) and in bacteria (Starr, DeLey & Kilgore, 1957). The reaction is believed to be the first step in the conversion of D-glucuronate into L-ascorbate in animal tissues. The reduction of the uronic acids is interesting because of their structural similarity to the hexose phosphates, which are also reduced in the presence of TPNH and silkworm haemolymph (Faulkner, 1956*b*) at rates which are of the same order as for D-glyceraldehyde.

These specificity studies indicate that sugars having two to four carbon atoms are rapidly reduced and there is a considerable fall off in rate with the pentoses and no reduction of the unsubstituted hexoses. However, if the pentoses and hexoses are substituted in their terminal carbons by an acidic group they are reduced rapidly. From the results given in Table 2 it appears that carbonyl compounds are reduced if the adjacent carbon is unsaturated or is substituted with an hydroxyl group or is part of another carbonyl group.

The enzyme in haemolymph also differs in its properties from the DPN-linked polyol dehydrogenases of rat liver, *Aerobacter suboxydans*, *Candida utilis* and *Pseudomonas* species, the substrate specificities of which have been extensively studied by McCorkindale & Edson (1954), by Arcus & Edson (1956) and by Shaw (1956). These enzymes oxidize hexitols and ribitols readily and attack erythritol and glycerol slowly or not at all.

The evidence presented here indicates that sugar phosphates and the simpler carbonyl compounds are reduced by the same enzyme in the haemolymph, but the results have to be interpreted with caution until the fraction containing the reducing activity has been purified further.

The actual role that the polyol dehydrogenase of *Bombyx mori* tissues plays in the physiology of the insect will remain in doubt until more is learned of the chemical composition of the haemolymph and other tissues. The present studies have indicated the class of compound that may be reduced in the insect, but the 'natural' substrates are not yet known. It is significant that Wyatt & Kalf (1958) reported finding glycerol in the haemolymph of the giant silkworm (*Platysamia cecropia* L.), and the possibility should now be considered that glycerol is formed *in situ* by reduction of glyceraldehyde in the haemolymph. However, these studies emphasize the fact that active dehydrogenases are present in the haemolymph and add weight to the suggestion that the 'malic' enzyme and other dehydrogenases of the haemolymph make an important contribution to the maintenance of the redox potential (Dennell, 1949; Faulkner, 1956*a*).

SUMMARY

1. Dialysed haemolymph of the silkworm, *Bombyx mori* L., contains a triphosphopyridine nucleotide-linked polyol dehydrogenase that reduces glycolaldehyde to ethylene glycol, DL-glyceraldehyde to glycerol and D-erythrose to erythritol. Among the other hydroxyaldehydes and carbonyl compounds reduced by the enzyme preparation are D-threose, glucuronic and galacturonic acids, ribose 5-phosphate, glyoxal, methylglyoxal and dimethylglyoxal. Hexoses and pentoses are not reduced and dihydroxyacetone is reduced at 5% of the rate for D-glyceraldehyde.

2. The equilibrium of the reaction favours the reduction of the carbonyl compounds at pH 7·5; but the oxidation of butane-2:3-diol, propylene glycol and glycerol in the presence of triphosphopyridine nucleotide can be demonstrated at high substrate concentrations (M) at pH 8·5.

3. The enzyme oxidizing the glycols is distinct from the 'malic' enzyme of the haemolymph, but the reduction of glyceraldehyde and ribose 5-phosphate appears to be catalysed by the same protein fraction.

4. Polyol dehydrogenase has been detected in extracts of the fat body and the gut of larvae, and in extracts of whole pupae and adults.

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Phospholipids

5. THE EFFECT OF COD-LIVER OIL IN THE DIET ON THE COMPOSITION OF HEN'S EGG PHOSPHOLIPIDS*

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The degree of unsaturation of the lipids of the hen's egg is known to be influenced by the composition of the fats present in the diet (Henriques & Hansen, 1903; McCollum, Halkin & Drescher, 1912; Terroine & Belin, 1927). Cruickshank (1934) found that the effect of a change in the dietary fat reached its maximum after about 16 days, reflecting the time taken for the laying down of the yolk in the oviduct. Although the iodine value of the yolk lipids could be increased greatly by feeding highly unsaturated oils such as linseed or hempseed, relatively saturated fats such as mutton fat or palm oil had only a slight depressing effect, and foreign saturated acids such as lauric and myristic were not transmitted to the yolk.

Reiser (1951) added 2% of cod-liver oil to a fat-free feed and found evidence of pentaene and hexaene structures in the fatty acids of the yolk lipids by spectrophotometric analysis. On a rigorously fat-free diet hexaethenoid acids were found to disappear completely from the egg lipids, and the proportions of the other polyethenoid acids diminished; however, the total amount of unsaturated acids was not affected (Reiser, Gibson, Carr & Lamp, 1951). These workers examined either the total lipids of the yolk, or the glycerides and phospholipids as separated by precipitation with acetone.

* Part 4: Rhodes & Lea (1957).

It has recently been shown (Hanahan, 1954; Rhodes & Lea, 1956*a*) that the fatty acid in the α' position of the phosphatidylcholine (lecithin) and of the phosphatidylethanolamine of normal eggs is apparently always unsaturated, whereas that in the β position is almost entirely saturated. Moreover, the unsaturated acids of the phosphatidylethanolamine and of a small fraction of the phosphatidylcholine possessed unusually high average unsaturation for an animal lipid of non-marine origin (Rhodes & Lea, 1956*a*). The hens had been receiving a normal diet which, however, contained some fish meal.

The object of the present work was to ascertain whether the highly selective deposition of unsaturated and saturated acids in the α' and β positions of the yolk phospholipids would persist when a high proportion of fish oil, rich in polyethenoid acids, was included in the diet.

EXPERIMENTAL

Materials

Basic ration. This was a commercial laying meal containing 5% of white-fish meal.

Defatted meal. The meal (1 kg.) was refluxed with diethyl ether (2 l.) for 2 hr., filtered off and the residue re-extracted with fresh solvent. The final residue was dried and supplemented with 5 mg. of β-carotene and 22 mg. of α-tocopherol/kg.

Cod-liver oil and the methyl esters of its more unsaturated acids. The fresh medicinal cod-liver oil used had an iodine value of 167, corresponding to a mean unsaturation of approximately 6·9 m-moles of double bond/g. or 2 double bonds/mol. of its constituent fatty acids. The acids were obtained by alkaline hydrolysis and were fractionated by the method of Silk, Sephton & Hahn (1954), with precautions against atmospheric oxidation. The more unsaturated acids recovered from the acetone-soluble lithium salts represented 28% of the total, and had an average unsaturation of 12·3 m-moles of double bond/g., equivalent to approximately 4 double bonds/mol. A repetition of the lithium salt separation raised the unsaturation only to 12·9 m-moles/g. with a yield of 44%, and a single precipitation was therefore used in the main preparation. After esterification with methanol the resulting esters of the more unsaturated acids were dissolved in light petroleum (b.p. 40–60°) and passed through a column of alumina to remove any products of autoxidation present; the solvent was then removed as completely as possible.

Methods

Feeding. White Leghorn × Rhode Island Red pullets were fed with meal (basic ration) and water *ad lib.* When additions were made to the feed the oil was mixed daily with 100 g. of the meal, and when this had been consumed a further 100 g. of plain meal was given, part of which was left uneaten. Cod-liver oil in amounts of 10 and 30 g. daily, representing 14 and 34% of the total calories respectively, was readily accepted by the hens when given in this way, and had no effect on appetite or frequency of laying.

The defatted meal was not well accepted, however, and when the esters of the highly unsaturated acids were included the feed was refused almost entirely and the birds ceased to lay, probably because of the low intake of food. Methyl esters of tallow fatty acids have been fed to chicks in amounts up to 15% of the ration without evidence of toxicity (Reiser, Dieckert & Hamilton, 1956). The odour of the unsaturated esters was indistinguishable from that of the oil and no obvious explanation for their rejection could be found.

Analytical. The general analytical methods used have been described previously (Rhodes & Lea, 1957).

Examination of the yolk lipids. Eggs were collected daily and the yolks freeze-dried. The lipid of a single yolk was extracted by shaking the dried material twice with 50 ml. portions of chloroform–methanol (1:1, v/v) at 0° for 30 min. under nitrogen. The combined extracts were evaporated to dryness and the total lipid was applied as a 5% (w/v) solution in chloroform–methanol (98:2, v/v) to a column of 10 g. of silicic acid. The column was washed with the same solvent to complete the elution of the neutral fat, and the adsorbed phospholipids were recovered by elution with methanol–chloroform–water (75:20:5, by vol.).

The phosphatidylcholine and phosphatidylethanolamine were separated by chromatography on silicic acid in chloroform–methanol (70:30, v/v) as previously described (Rhodes & Lea, 1957).

The component fatty acid in the α' position and the corresponding lyso-compound were obtained from each phospholipid by the action of the phospholipase *A* of snake venom (*Agkistrodon piscivorus piscivorus*) by the technique of Hanahan, Rodbell & Turner (1954).

RESULTS

Experiment 1

One bird, laying regularly on the basic ration, was given 10 g. of cod-liver oil daily for 19 days, followed by 30 g. of oil daily for 15 days, and was then put on defatted meal for a further 19 days. Over the whole period the hen laid an average of 5·4 eggs/week. The results of the analyses of the lipids of the eggs examined (Table 1) confirmed the

Table 1. *Effect of diet on the unsaturation of hen's egg phospholipids* (*Expt.* 1)

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

Days on diet	Neutral fat (iodine value)	Lipid phosphorus/ yolk (mg.)	Unsaturation (double bonds/atom of P)				
			Total phospholipid	PC	PE	LysoPC	LysoPE
			(*a*) Feed included 10 g. of cod-liver oil/day				
0	73·6	60·7	2·26	2·00	3·64	0·16	0·42
1	75·0	59·8	2·28	—	—	—	—
4	76·5	60·9	2·51	—	—	—	—
8	79·0	56·2	3·05	—	—	—	—
12	79·8	56·9	2·98	—	—	—	—
19	83·7	58·4	3·24	2·75	4·77	0·18	0·35
			(*b*) Feed included 30 g. of cod-liver oil/day				
8	83·4	56·2	3·25	—	—	—	—
13	84·7	50·3	3·69	3·10	5·23	0·25	0·40
14	88·9	49·4	3·53				
			(*c*) Fed defatted meal				
10	75·8	62·3	2·91	—	—	—	—
19	74·7	66·4	2·42	—	—	—	—
			(*d*) Fed more highly unsaturated esters				
			No eggs after 5 days feeding				

findings of Cruickshank (1934) that about 2 weeks are required to bring the egg lipids into equilibrium with the dietary fat.

The addition of 10 g. of cod-liver oil daily (Table 1*a*) raised the mean unsaturation of the glycerides by 14% and that of the total phospholipids by 43%. The gross increase in the unsaturation of the phosphatidylethanolamine was greater than that of the phosphatidylcholine (1·13 and 0·75 double bonds/atom of P respectively), but the percentage increases were almost the same (31 and 37%). No change was found in the slight unsaturation of the derived lyso-compounds, that is of the component β-fatty acid of the diacylphospholipids, indicating that the increase in unsaturation had been confined to the acids in the α' position of both phospholipids.

When the rate of feeding of the oil was raised to 30 g./day (Table 1*b*), the mean unsaturation increased further, the highest levels reached corresponding to increases of 21% for the glycerides, 56% for the total phospholipids, 44% for the phosphatidylethanolamine and 55% for the phosphatidylcholine. Even at these levels the increases in unsaturation were still confined to the α' acids of the phospholipids.

The distribution of the polyethenoid acids in the individual phospholipids was further examined by subdivision of the peaks eluted from the silicic acid columns. The more unsaturated components appeared in the earlier fractions, as had previously been found (Rhodes & Lea, 1956*b*). The recovery of the phospholipid P of the phosphatidylcholine (eggs *b*13 and *b*14, Table 1) in fractions within given ranges of unsaturation was compared with similar figures for the phosphatidylcholine from a normal egg (Table 2). It appears from these data that the polyethenoid acids from the fish oil had been laid down in the phosphatidylcholine largely at the expense of the major proportion of diethenoid and monoethenoid acids normally present.

A similar fractionation of the more rapidly running phosphatidylethanolamine peak was not carried out, but it is clear from Table 1 that the α' acids, which reached a mean unsaturation of over 4·8 double bonds/molecule, cannot have contained more than a small proportion of monoethenoid or diethenoid component acids.

When the hen was returned to a fat-free diet (Table 1*c*), the unsaturation of the egg lipids rapidly fell towards the normal value.

Table 2. *Fractionation of egg phosphatidylcholines, according to unsaturation, by chromatography on silicic acid columns*

Unsaturation limits (double bonds/atom of P)	Lipid phosphorus recovered (%)		
	Normal feed	Cod-liver oil supplement	Diff.
Above 5	1·1	14·4	+13·3
4–5	5·8	20·6	+14·8
3–4	14·8	19·5	+ 4·7
2–3	21·8	25·8	+ 4·0
Below 2	56·5	19·7	−36·8

Experiment 2

The inclusion of the more-highly unsaturated esters at the end of Expt. 1 (Table 1*d*) coincided with the cessation of egg production. A fresh pullet was used, therefore, to compare the effect of 10 g. of these esters in place of the whole oil. Use of the esters nearly doubled the mean unsaturation of the unsaturated acids in the dietary supplement, and increased the net weight of the highly unsaturated acids fed by a factor of four.

The feed containing the esters was only partly consumed, and after four eggs had been laid in 5 days one more was obtained on the twelfth day. After 20 days the bird was killed and the contents of the oviduct were examined. There were 19 yolks present, the largest being about 0·9 cm. in diameter. These were dried and processed together.

The analyses showed: neutral fat 300 mg., iodine value 105; phospholipid 65 mg., total P 2·60 mg., unsaturation 3·72 double bonds/atom of P. When separated, the phosphatidylethanolamine fraction comprised 59% of the lipid P, of unsaturation 4·39 double bonds/atom of P; and the phosphatidylcholine 41%, of unsaturation 2·89 double bonds/atom of P. These levels of unsaturation were close to those found in complete eggs from the hen fed the basic ration supplemented with cod-liver oil (Table 1; *a*19, *b*13, *b*14) but the proportion of phosphatidylethanolamine in these early stages of development was much higher than the figure of $<20\%$ found in the entire egg (Rhodes & Lea, 1957).

Experiment 3

In order to obtain larger amounts of the highly unsaturated phospholipids a third hen was fed with the basic ration containing 15 g. of cod-liver oil daily for 41 days. The composition of the lipids of the eggs responded to the change of feed in the same general way as in Expt. 1 (Table 3).

Isolation of the α' acids. Phosphatidylethanolamine, prepared from the combined six eggs (Table 3) containing 28·0 mg. of lipid P (0·90 m-mole), was dissolved in 200 ml. of diethyl ether and treated with 5 mg. of venom in 5 ml. of aqueous 0·005 M-calcium chloride solution, and 0·5 m-mole of ammonia in ether (Long & Penny, 1957). A considerable precipitate formed within 2 hr., and after 4 hr. the solvent was removed by evaporation under reduced pressure. The residue was dissolved in 5 ml. of chloroform and applied to a column of

Table 3. *Effect of feeding 15 g. of cod-liver oil/day on the unsaturation of the egg lipids (Expt. 3)*

Days on diet	Neutral fat (iodine value)	Total lipid phosphorus (mg.)	Unsaturation (double bonds/atom of P)		
			Total phospholipid	PC	PE
0	72·6	61·0	2·33	—	—
7	78·5	72·2	2·87	—	—
15	84·7	68·2	3·04	2·70	4·79
27, 28, 30, 32, 34, 35	86·7	340	3·15	2·71	4·81

5 g. of silicic acid. Elution was continued with chloroform and the effluent collected. In all of these manipulations an atmosphere of nitrogen was maintained over the solutions.

After removal of the solvent 250 mg. (0·83 m-mole) of colourless liquid acids was recovered, free of phosphorus, iodine value 374, corresponding to an average unsaturation of approximately 4·4 double bonds/mole. The α' acids were recovered, therefore, in good yield and with the expected degree of unsaturation.

The α' acids were similarly obtained from phosphatidylcholines.

DISCUSSION

The cod-liver oil fed in these experiments had an iodine value of 167, equivalent to 6·58 m-moles of double bond/g. Assuming the presence of 16% of saturated acids (Hilditch, 1947) and a mean molecular weight of 300 for the unsaturated acids, the mean unsaturation of the unsaturated acids of the oil was of the order of 2·4 double bonds/molecule.

In Expt. 1 a total of 65·8 m-moles of double bond was fed in the 10 g. of oil/day, and the maximum increases in the unsaturation of the egg lipids when in equilibrium with this feed amounted to 0·40 m-mole of double bond/g. of glyceride and 0·98 m-mole/m-mole of phospholipid (Table 1*a*). The increase in the total unsaturation of an egg of average size would therefore be of the order of 4 m-moles, and, if laid daily, this represents no more than 6% of the unsaturation fed. It is not surprising, therefore, that trebling the level of oil fed (Table 1*b*), or the substitution of the esters of the more highly unsaturated acids for the whole oil, produced only a slight further increase in the average unsaturation of the yolk lipids.

The synthetic pathway from the ingested fat to the egg phospholipid appeared to be selective for the highly unsaturated acids in the feed; the average unsaturation of the α' acids of the phosphatidylethanolamine, for example, at 3·2 double bonds/mol., was considerably higher than that of the total acids of the oil (2·0 double bonds/mol.) or of the unsaturated acids in the oil (2·4 double bonds/mol.), but nevertheless rose markedly when the oil was fed. Furthermore, the polyethenoid acids of the feed were incorporated into the two phospholipids approximately in proportion to their initial unsaturation, the ratio of the unsaturation in the α' position of the phosphatidylethanolamine to that in the phosphatidylcholine being 1·75 on the normal diet, and remaining at 1·72 and 1·70 after feeding the oil (eggs *a* 0, *a* 19 and *b* 13 and *b* 14, Table 1).

Reiser *et al.* (1951) found that the laying hen on a fat-free diet is able to maintain the total level of unsaturated acids in the egg, including some 'essential' diethenoid acid, by synthesis from other carbon sources, the polyethenoid acids present on a normal diet being replaced by monoethenoid. In the present work only part of the less highly unsaturated acids was replaced by the polyethenoid acids fed, even when a large excess was present in the diet. The mono- and di-ethenoid acids were almost eliminated from the phosphatidylethanolamine, but considerable quantities remained in the phosphatidylcholine. The ease of interchangeability of the unsaturated fatty acids of the egg phospholipids is in agreement with the observations of Hanahan (1956), who concluded that the unsaturated acid in the α' position of the lecithins of rat liver was in a metabolically more reactive site than the saturated acid in the β position.

The considerable difference between the compositions of the unsaturated fatty acids in the α' positions of the two glycerophospholipids of the egg, which was maintained throughout the changes induced by the experimental diets, affords no support for a biosynthetic route to these compounds in the laying hen involving a common precursor, such as the reaction of an $\alpha\beta$-diglyceride with the cytidine diphosphate derivative of choline or ethanolamine, or an unspecific interchange of the basic alcohols of the completed molecule (Kennedy, 1956).

SUMMARY

1. The addition of 10 g. of cod-liver oil/day to the diet of a laying hen increased the unsaturation of the neutral fat and more particularly of the phospholipids of the egg yolk.

2. The increases in unsaturation of the egg phosphatidylcholine and phosphatidylethanolamine were confined entirely to the fatty acids esterified in the α' positions of the glycerophospholipids, the β-acid remaining mainly saturated.

3. The polyethenoid acids of the fish oil were selectively incorporated into the phospholipids apparently at the expense of the monoethenoid and diethenoid acids normally present. An average unsaturation of 4·8 double bonds/mol. was reached in the α' acids of phosphatidylethanolamine.

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